(Exhibit 32)

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(12) **EUROPEAN PATENT APPLICATION**

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(54) Displacement polynucleotide assay method and polynucleotide complex reagent therefor.

(57) A diagnostic reagent is disclosed containing a complex of a probe polynucleotide (P) bound via purine/pyrimidine hydrogen bonding to a labeled polynucleotide (L). The probe (P) contains a target binding region (TBR) capable of binding to a target nucleotide sequence (G) of a biological sample. A method is disclosed in which contact with a sample containing the target nucleotide sequence (G) causes binding, initially between G and a single-stranded portion (IBR) of the target binding region (TBR). Thereafter the labeled polynucleotide (L) is displaced from the complex by branch migration of (G) into the (P)/(L) binding region. Determination of displaced labeled polynucleotide (L) gives a value which is a function of the presence and concentration of target nucleotide sequence (G) in the sample.
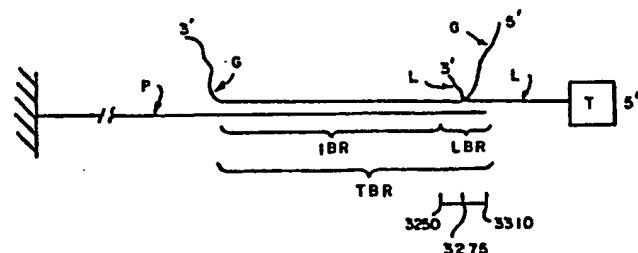
FIG.1C

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DISPLACEMENT POLYNUCLEOTIDE ASSAY METHOD AND POLYNUCLEOTIDE COMPLEX REAGENT THEREFOR

BACKGROUND OF THE INVENTION

The present invention relates to a diagnostic assay method for detecting the presence of a target nucleotide sequence (either DNA or RNA) in a biological sample, and to a polynucleotide reagent complex therefor.

Conventional methods for detecting the presence of a particular polynucleotide in a biological sample typically involve immobilization of nucleic acid of the sample on a surface as the initial step. Once the sample is immobilized, a probe polynucleotide strand, tagged usually with a detectable label such as radioactive phosphorus atoms, is incubated with the immobilized sample so as to bind to the immobilized sample by purine/pyrimidine base sequence-specific complementary base pairing when the immobilized sample contains the target nucleotide sequence. After washing off the labeled probe which has not so hybridized, the presence or absence of label on the support is then determined. Techniques for this determination include exposure of a photographic film, liquid scintillation counting, and fluorescence microscopy.

Ward and coworkers (see EPA 63,879 (1982)) have described a variation of this technique in which, rather than tagging the probe directly with a detectable label, the probe is tagged with a nonisotopic substituent such as biotin on certain nucleotides. In such case, after the unhybridized probe is washed off, the support is contacted with a reagent such as avidin linked to an enzyme. The avidin-enzyme complex binds selectively to biotin because of the high avidin-biotin binding affinity, so as to affix enzyme selectively where the target nucleotide sequence has been immobilized on the substrate. Thereafter, a substrate for the enzyme is added and products of the enzymatic reaction are detected, yielding an amplified signal functionally dependent upon the initial concentration of target nucleotide sequence

on the substrate. See also EPA 97,373 of ENZO BIOCHEM, INC (January 4, 1984).

A variation in the above nonisotopic system has also been described in another European patent application of Standard Oil of Illinois (EPA 70,687 (1983)) in which, in one form (see pages 8-10 thereof), two probes specific for the target nucleotide sequence are employed. The first probe, which can hybridize to a first portion of the target nucleotide sequence, is affixed to a solid support such that, upon incubation of the solid support with a sample of the biological material, target nucleotide sequences in the sample will bind to the support selectively via this first immobilized probe. Thereafter, the second probe, which can hybridize selectively to a second and distinct portion of the target nucleotide sequence, is used to contact the support. Again, if the target nucleotide sequence had been present in the biological sample, the second probe will now bind selectively to that nucleotide sequence; and a combination structure (or sandwich) will be created linking the second probe to the support via the first probe and the target nucleotide sequence. The reference discloses labeling this second probe with a moiety directly or indirectly generating or absorbing specific wavelengths of light (e.g., a fluorescent label, a phosphorescent label or a chemiluminescent label). By separating the support from unbound solution constituents at each stage, the presence of label in the phase with support after the third separation will be a function of the presence and concentration of the target nucleotide sequence in the sample. See also WO 83/01459 of Orion-Yhtma Oy (April 29, 1983).

A different diagnostic method for a specific target nucleotide involving digestion of double-stranded sample nucleic acid in solution with a restriction enzyme, followed by detection of specifically sized fragments on filter paper, is disclosed in U.S. Patent 4,395,486 to Wilson et al. In that disclosure, the presence of the

single base substitution causative of sickle cell anemia
abolishes a specific site for restriction enzyme cleav-
age, and thereafter two specifically sized small frag-
ments which are usually detected are then detected in
reduced amounts (for sickle cell trait) or cease to be
detected (for sickle cell anemia).

While the above procedures will detect the presence
of nucleotide sequences in biological samples in many
cases, they each have the disadvantage of either multi-
ple steps or steps with necessarily long incubation
periods that make them impracticable for easy use in a
clinical laboratory. Furthermore, many of these pro-
cesses suffer from a limited selectivity or sensitivity
with regard to interfering polynucleotide sequences or
the detection of low levels of target nucleotide
sequence reliably against the background signal. In
particular, nonspecific binding of the labeled probe
represents a source of substantial background signal in
each process.

Apart from the analysis of biological samples for
target nucleotide sequences, various aspects of the
physical chemistry of hybridization (formation of
double-stranded helices between complementary poly-
nucleotide sequences) have been studied. These studies
have included examination of the phenomena of strand
migration and displacement in nucleic acid, both _in vivo_
and _in vitro_. By referring to such studies, however, we
do not admit that the phenomena of strand migration and
displacement have any obvious applicability to diagnosis
and detection. C. Green and C. Tibbetts, _Nucleic Acids
Research_ vol. 9, No. 8, pp. 1905-18 (1981), have des-
cribed the formation of a complex (hybrid) of a 6.1 kb
(6100 base long) single-stranded DNA polynucleotide
hybridized near its middle (the interval 1.7 - 3.3 kb)
by an end-labeled complementary DNA polynucleotide of
1.6 kb length. Addition to this complex, in solution,
of the 6.1 kb complementary strand caused rapid dis-
placement of the labeled polynucleotide (see Fig. 2 on

page 1910 of this reference), monitored by taking aliquots of the reaction mixture, separating then by gel chromatography and analyzing then by autoradiography. The displaced 1.6 kb polynucleotide increased steadily from under 10% to over 90% of the radioactive signal in a period of more than 85 minutes (depending upon concentration) with the 1.6/6.1 kb hybrid accounting for the bulk of the remaining radioactivity. The presumed partially displaced intermediate, which would have a total mass equivalent to 13.8 kb of DNA (both long strands and a partially displaced short strand) was apparently not detected. The authors concluded that the initial hybridization of the two 6.1 kb polynucleotides, forming a branched species, was the rate-limiting step; and that displacement along the 1.6 kb paired region of a labeled polynucleotide was very rapid, consistent with a calculated average lifetime of the branched (13.8 kb mass equivalent) species of 0.8 minutes. They indicate the possibility of both single-branched or doubly nucleated (D-looped) intermediate species (illustrated on page 1912 of the reference). In order to better study the phenomenon of branch migration, they attempted to slow the displacement process, by using drugs which might retard the migration phenomenon and/or by using complexes with more than 1.6 kb of hybrid base pairing (see pages 1913-1914 of the reference). It should be noted that the 1.6/6.1 kb species was challenged by Green et al only with the 6.1 kb complementary strand, purified away from any non-specific strands.

A separate issue in nucleic acid biochemistry has been the examination of polymeric species which interact with nucleic acids during strand hydridization processes. Polyethers such as poly(ethylene glycol) have found use in a variety of specific biological or biochemical processes such as viral particle isolation (K.R. Yamamoto et al., Virology vol. 40, pp. 734 - 744 (1970)), nucleic acid purification (B. Alberts in Methods in Enzymology, S.P. Colowick and N.O. Kaplans,

eds. (Academic Press, N.Y.), vol. 12, pp. 566-581
(1967)), protein purification (K.G. Ingram, <u>Arch.
Biochem. Biophys</u>. Vol. 194, pp. 59-68 (1977)), mammalian
cell fusion (G. Galfre et al., <u>Nature</u> vol. 266, pp. 550-
552 (1977)) and enhancement of specific enzymatic
activity (B.H. Pfeiffer and S.B. Zimmerman, <u>Nucleic
Acids Research</u> vol. 11, pp. 7853-7871 (1983)). M. Renz
and C. Kurz, <u>Nucleic Acids Research</u>, vol. 12, 3435-44
(1984) (which may not consitute prior art with respect
to the present invention) disclose the use of
polyethylene glycol as a volume excluding agent to
enhance the rate of hybridization in an immobilized-
sample type of DNA probe assay (particularly of the type
referred to as a Southern blot). See especially page
3441 of this reference. In this respect other volume
exclusion polymers of various types have been shown to
enhance the rate of hybridization of matched single
strands. See Wetmur et al., <u>Bipolymers</u>, vol. 10, p. 601
(1971) (dextran sulfate), Wahl et al., Proc. Nat. Acad.
Sci., vol. 76, p. 3683 (dextran sulfate). In general,
polymers useful in this regard are non-ionic or anionic
water-soluble polymers (including polysaccharides) which
do not react with DNA. See S.B. Zimmerman et al, <u>Proc.
Nat. Acad. Sci</u>., vol. 80, pp. 5852-56 (1983); B.H.
Pfeiffer et al, <u>cited above; Water-soluble Synthetic
Polymers</u> (P. Molyneux, ed.; CRC Press, Inc., Cleveland,
OH — two volumes, 1983).

BRIEF DESCRIPTION OF THE INVENTION

The present invention is based upon the rapid dis-
placement of a labeled polynucleotide from a probe poly-
nucleotide by the target nucleotide sequence of a sam-
ple, enabling direct or indirect measurement of label
found in or on the displaced labeled polynucleotide (or
in some cases the labeled polynucleotide not dis-
placed). This label serves as a reliable and quantita-
tive measurement functionally related to the presence
and concentration of target nucleotide sequence in a
sample. Accordingly, the present invention includes a

method for determining the presence of a predetermined
target nucleotide sequence (either DNA or RNA) in the
nucleic acid of a biological sample which comprises the
steps:

(a)   providing a reagent complex of (i) a probe
polynucleotide which is capable of base pair binding via
hydrogen bonds of purine/pyrimidine base pairs to the
target nucleotide sequence, and (ii) a labeled poly-
nucleotide which is bound by base pair binding via
hydrogen bonds of purine/pyrimidine base pairs to the
probe polynucleotide in a region of the probe poly-
nucleotide at least partially coextensive with the
region in which the probe polynucleotide is capable of
binding to the target nucleotide sequence;

(b)   contacting the reagent complex with a sample
under conditions in which the target nucleotide
sequence, if present, binds to the probe polynucleotide
and displaces labeled polynucleotide from the reagent
complex; and

(c)   determining the presence (which includes of
course determining the amount) either of labeled poly-
nucleotide displaced from the reagent complex or of the
labeled polynucleotide remaining in the reagent complex.

The present invention also includes a diagnostic
reagent for determining the presence of a target nucleo-
tide sequence in the nucleic acid of a biological sample
comprising the reagent complex of:

(i) a probe polynucleotide which is capable of
binding via hydrogen bonds of purine/pyrimidine base
pairs to the target nucleotide sequence, and

(ii) a labeled polynucleotide which is bound via
hydrogen bonds of purine/pyrimidine base pairs to the
probe polynucleotide in a region of the probe poly-
nucleotide at least partially coextensive with the
region in which the probe polynucleotide is capable of
binding to the target nucleotide sequence;

the base pairing between the target nucleotide
sequence and the probe polynucleotide being of

sufficient cumulative binding strength (as defined
below) for the target nucleotide sequence, if present in
a sample with which the reagent is contacted, to be able
to displace labeled polynucleotide from the reagent
complex.

The present invention further includes such a
method or a diagnostic kit containing such a reagent
complex wherein a volume excluding inert polymer which
is non-ionic or anionic (but which is preferably, but
not necessarily a polyether) is present in such amounts
sufficient to increase the rate of appearance of
displaced labeled polynucleotide.

This effect, increasing the rate of appearance of
specifically displaced polynucleotide, is sometimes
referred to in the description that follows as an
increasing rate of displacement. In such event, that
nomenclature should not be read as implying that the
actual displacement step is expedited on a microscopic
or molecular level. In fact, Applicants currently
believe that the inital nucleation stage in
hybridization of competitor strand to the probe
polynucleotide (see Figures 1A and 1B) is the rate-
limiting step which is enhanced by volume excluding
polymer. If that is true, then no enhancement of the
displacement step (cf. Figures 1C, 1D and 1E) is
required on a microscopic or molecular level to achieve
an increase in the overall rate (compare Figure 1A to
Figure 1E).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic view of one embodiment of
the reagent complex of the present reagent useful in the
present process;

Figure 1B is a view similar to Figure 1A in which
the reagent complex is hybridized with a target nucleo-
tide sequence G of sample nucleic acid;

Figure 1C is a view similar to Figure 1B in which
the sample nucleic acid has begun to displace labeled
polynucleotide from the reagent complex;

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Figure 1D is an enlarged view similar to Figure 1C, in which the helical structure of double-stranded portions is schematically shown;

Figure 1E is a view similar to Figure 1C in which the labeled polynucleotide has been fully displaced from the reagent complex;

Figure 1F is a view similar to Figure 1B of a second embodiment of the present invention in which the immobilized polynucleotide has sequences complementary to and base paired with the sample nucleic acid on both sides of the region where the labeled polynucleotide is bound;

Figure 1G is a view similar to Figure 1A of a reagent complex according to a modification of the first embodiment.

Figure 2 is a view similar to Figure 1A of a reagent complex according to a third embodiment of the present invention, with a sample polynucleotide having the target nucleotide sequence shown prior to hybridization to the reagent complex;

Figure 3A is a view similar to Figure 1A of a reagent complex according to a fourth embodiment of the present invention;

Figure 3B is a view similar to Figure 1C of the fourth embodiment of Figure 3A.

Figure 3C is a view similar to Figure 1E of the fourth embodiment of Figure 3A.

Figure 3D is a view similar to Figure 3A of a modified form of the reagent complex of the fourth embodiment.

Figure 4 is a view similar to Figure 1A of a reagent complex according to a fifth embodiment of the present invention;

Figure 5 is a view similar to Figure 1A of a reagent complex according to a sixth embodiment of the present invention; and

Figure 6 is a view similar to Figure 1A of a reagent complex according to a seventh embodiment of the

present invention.

DETAILED DESCRIPTION OF THE INVENTION

In this application the following terms are used based on their generally accepted meanings in the field of molecular biology:

Polynucleotide or Polynucleotide Strand refers to a linear polymeric structure of pentose sugars (generally ribose or deoxyribose) linked to each other by 3',5'-phosphodiester linkages, and linked by carbon-nitrogen bonds at the 1-carbon of the sugar to pendant purine or pyrimidine bases such as, but not limited to, uracil (linked naturally to ribose only as rU), thymine (linked naturally to deoxyribose only as dT), cytosine (dC or rC), adenine (dA or rA) and guanine (dG or rG). Polynucleotides thus include strands of deoxyribonucleic acid (DNA) and strands of ribonucleic acid (RNA).

The ends of such Polynucleotide Strands are referred to as the Five Prime (5') end, where the 5-carbon of the pentose is not linked to another pentose (but may bear hydroxyl, monophosphate or other natural or synthetic moieties), and the Three Prime (3') end, where the 3-carbon of the pentose is not linked to another pentose (but may similarly bear hydroxyl, monophosphate or other natural or synthetic moieties).

Complementary Base Pairing or Purine/Pyrimidine Base Pairing refers to the hydrogen bonding between opposite bases pendant on two antiparallel Polynucleotide Strands, which is most energetically favorable for natural DNA when dG is opposite dC and dA is opposite dT. Bases other than the five naturally-prevalent ones also have preferential pairing: for example, 5-methylcytosine binds preferentially to guanine. For illustrative purposes, this pairing is shown in many of the Figures by parallel straight lines with complementary strands directed in antiparallel directions (in the 5' to 3' sense). It should be appreciated, however, that the actual geometry of double-stranded segments will normally be helical (the well-known double helix) of

various pitches, as schematically illustrated in Figure
1D.

Hybridization is used herein to refer to admixing
two Polynucleotides under conditions conducive to the
formation of double-stranded structures, with Complemen-
tary Base Pairing causing such double stranded struc-
tures to form where complementary sequences or nearly
complementary sequences are present.

The basic components of the method of the invention
are a probe polynucleotide (sometimes called herein the
probe), a labeled polynucleotide (sometimes called
herein tagged polynucleotide or release tag), and the
biological sample containing nucleic acid, a portion of
which is sometimes called herein the target polynucleo-
tide or target nucleotide sequence. A sample may or may
not contain a target nucleotide sequence. In some cases
a volume excluding polymer such as a polyether compound
is also provided. In some cases a support is also
provided, either to which the reagent complex is
immobilized via the probe (such that the probe is
sometimes called an immobilized probe or immobilized
probe polynucleotide), or in other cases as a part of
the separation step that may follow displacement as a
part of the determination or detecting step. In prac-
ticing the process, additional reagents or equipment are
frequently required for readout; the term readout refers
to the direct or indirect detection of labeled
polynucleotide in one or more phases of (usually
separated) reaction materials, and especially in a
liquid phase by virtue of displacement from the reagent
complex and separation of displaced labeled polynucleo-
tide in solution from probe polynucleotides and reagent
complexes.

In the practice of the present invention, the probe
polynucleotide can be a linear or circular polynucleo-
tide capable of binding specifically through complemen-
tary base pairing in at least one region of its purine/
pyrimidine base sequence to specific target nucleotide

sequences of a sample. This binding may be between DNA
and RNA, between DNA and DNA or between RNA and RNA.
Accordingly, the probe may either be DNA or RNA. As
discussed more fully below, it is generally only a spe-
cific region of the probe which binds selectively to the
target nucleotide sequence. Other regions of the probe
may be of various naturally occurring or synthesized
sequences which do not participate in the hybridization
reaction with the target nucleotide sequence, but which
may play an important role in the present invention,
e.g., by serving as a site for attachment to a support
or by providing some degree of separation between the
support and the region to which the target nucleotide
sequence binds, if desired.

Referring to the region of the probe to which the
target nucleotide will specifically bind, called herein
the target binding region (TBR in the Figures), the
binding may be (and preferably is) perfect, in the sense
that each nucleotide in the probe sequence finds its
correct complementary binding partner (e.g., dA to dT)
in the target nucleotide sequence, or may contain some
mismatches. At least one portion of the target binding
region of the probe is preferably single-stranded in the
reagent complex, i.e., it is not complementary to
labeled polynucleotide sequences nor self-complementary;
this single-stranded region is sometimes called herein
the initial binding region (IBR in the Figures) because
the target nucleotide sequence can bind to this region
of bases without displacing any of the labeled
polynucleotide. Such initial binding region of the
probe is at least fifteen bases in length, and is
preferably at least fifty bases in length. The overall
target binding region includes the initial binding
region and most or (preferably) all of the labeled
polynucleotide binding region (LBR in the Figures and in
the discussion below). The length of the overall target
binding region is not independently critical, but rather
can be considered as a function or sum of the preferred

or more preferred lengths of the IBR and LBR portions. Base lengths of the initial binding region of the probe above five hundred are generally not required, but are not significantly disadvantageous in most cases. A suitable lower limit on the length of this region of base pairing for clinical laboratory applications is somewhat dependent upon base sequence of the target binding region of the probe polynucleotide and base composition and other physical factors described below, and especially upon the conditions for intended hybridization, mode of attachment, if any, of the probe to a support, kinetics of hybridization and the readout system employed.

The solid phase to or on which the probe can be immobilized in certain embodiments, may be of almost any conventional type, including especially polymeric materials, ceramic materials, walls of a test tube or other container, paper, nitrocellulose or glass. In some forms of the invention, the solid phase consists of natural, modified natural or synthetic particles or beads made of materials such as protein, fixed cells or viruses, or various polymers such as polystyrene, latex or glass.

The means of attachment of the probe to the solid support in certain embodiments of the invention may be simple adsorption, but is preferably some form of specific covalent linkage, ionic bonding, hydrophobic interaction or hydrogen bonding. In the case of covalent linkage, the binding may be direct as by reaction between chemical moieties on the surface of the support (for example, amine or carboxyl moieties) and moieties on the polynucleotide, and especially hydroxyl or phosphate moieties on the end sugar rings of the polynucleotide. Linking agents which are specific to the free secondary hydroxyl normally present at the 3' end include phosphites, succinic anhydride and phthalamide. Linking agents which are specific to the phosphate normally present on the sugar at the 5' end

(at least for most naturally occurring polynucleotides
or products of most cleavage reactions) include carbodi-
imides such as 1-ethyl-3,3-dimethylaminopropylcarbodi-
imide, with or without imidazole or 1-methylimidazole.
See B.C.F. Chu et al., Nucleic Acids Research vol. 11,
pp. 6513-29 (1983). Such linkages which are specific to
an end or other small portion of the probe, if remote
from the target binding region, may permit greater
degrees of freedom during the hybridization reaction
compared to adsorption or other similar physical or non-
specific chemical means of attachment. With such
greater degrees of freedom, the minimum length of the
target binding region or minimum time for the
hybridization to progress to a detectable level may be
lowered.

Non-specific covalent linkages include linkages
between the substrate and free bases along the chain via
moieties such as m-aminobenzyloxy methyl (ABM), m-dia-
zobenzyloxy methyl (DBM) or o-aminophenylthioether
(APT). See H. Bunemann et al., Nucleic Acids Research
vol. 10, pp. 7163-7196 (1982) (two articles). Other
exemplary non-specific linking chemistry is described in
U.S. Patent 4,286,964 to B. Seed (1981).

In addition to direct covalent linkage, the probe
polynucleotide may be indirectly linked in a covalent
fashion to the support by a linking or spacer
molecule. Examples of indirect covalent linking
reagents include spacer reagents which can react by
carbodiimide chemistries both with functional groups
(for example, esters) on the support and with the
phosphate normally present on the 5' terminal sugar of
the polynucleotide. Such spacer molecules include the
aliphatic diamines used in the above-cited Chu et al.
article which, once attached to the terminal phosphate,
can then be linked to active groups on the support such
as N-hydroxysuccinimide esters. Other spacer molecules
include hydroxyalkanoic acids. Still other spacer
molecules can also contain a functional moiety such as

phenyl ketone which will react directly with a support
having hydrazine moieties, forming a resultant
hydrazone.

The probe further may be noncovalently linked to
the support by interaction of some portion of the probe
with affinity reagents that are adsorbed or covalently
bound to the support. Examples include (1) immobiliza-
tion on the support of a short single-stranded
polynucleotide which can hybridize to some portion of
the probe polynucleotide not overlapping with the region
in the probe which is capable of binding to the target
nucleotide sequence and (2) binding of a chemically
modified probe polynucleotide carrying one or more
avidin or biotin moieties to a support having biotin or
avidin moieties, respectively, adsorbed or covalently
bound to the support. The latter method is based on the
high affinity (K_{diss} approximately $10^{-15}M$) of the small
molecule biotin for the protein avidin (or
streptavidin).

While the present invention is not limited with
regard to the spacings between the point or points of
attachment of the probe to a support and the region of
the probe polynucleotide which binds specifically to the
target nucleotide sequence, it is preferable that this
spacing be sufficiently large for the hybridization
between target nucleotide sequence and target binding
region of the probe polynucleotide to occur such that
the target binding region of the probe has sufficient
and preferably maximal obtainable freedom of movement
during hybridization.

In other embodiments of the invention, the probe
polynucleotide is not immobilized on a support, but
rather the entire reagent complex is in solution as the
reagent is mixed with a biological sample such that
hybridization will occur, if at all, in solution. In
some of those solution hybridization embodiments, the
probe does contain a substituent (such as an affinity
reagent, e.g., biotin, or a chemical moiety, e.g., a

hapten such as nitrophenol) so as to be immobilizable or
separable if desired after hybridization, e.g., by
passing the reaction mixture through a porous bed or
filter that has streptavidin bound to the support
matrix. Such immobilization will cause only displaced
labeled polynucleotides to remain in the liquid phase,
for subsequent determination. Still other forms of the
invention involving solution hybridization include
alternative methods of separations such as size
exclusion chromatography (see Example 2 below),
electrophoresis (see, e.g., Examples 1 below), or other
physical separation techniques. Additional forms of the
invention, described more fully below in connection with
read-out, involve determination of displaced labeled
polynucleotide without any separation from complex. Of
course many of such determinations without separation
apply equally to processes wherein the complex includes
an immobilized probe polynucleotide.

Such probe polynucleotides can be manufactured
reproducibly in a variety of ways, e.g., cloned in or as
a part of a suitable plasmid or viral vector isolated
from a bacterium or other suitable microorganism, as a
part of the genetic material of a microbe or obtained
from any other pertinent biological source. Generally,
only a small region of nucleic acid that includes a
probe polynucleotide sequence (a portion of which forms
the target binding region) will be inserted into any
such cloning vectors by recombinant techniques; the
remainder, if any, of the cloned insert that is not
probe polynucleotide sequence can conveniently be chosen
from any polynucleotide sequence heterologous to the
target nucleotide sequence. In certain embodiments of
this invention, such heterologous sequences can include
sequences deliberately selected for specific properties
such as the presence of a unique restriction enzyme
recognition site. Under some conditions an entire gene
or a sequence including an entire gene may be used as an
insert, with the vector plus inserted nucleotide

sequence either in circular or linear form. In the
event that the probe polynucleotide is double-stranded
when manufactured, denaturation (either thermally, by
adjustment of pH or by disruption of base pairing with
other conventional techniques) will normally follow iso-
lation. Cleavage (especially by restriction enzymes or
by site-specific chemical cleavage) will normally be
used to form double-stranded segments of desired linear
form and, if double-stranded circular forms are grown,
will precede denaturation. In some cases it may be pre-
ferred to purify individual strands from a double-
stranded structure either to be used individually as
probe polynucleotides or with one as a probe
polynucleotide and the other as a precursor for the
labeled polynucleotide. This purification can be done
by standard techniques such as denaturing gel
electrophoresis or affinity chromatography. In some
instances, the probe polynucleotide is produced as a
single-stranded molecule by replication using single-
stranded vectors such as M13 bacteriophage. In some
other instances, as described below, the probe poly-
nucleotide and labeled polynucleotide can be
manufactured as parts of the same molecule.

The labeled polynucleotide used in the present
method and reagent is generally a smaller piece of
either DNA or RNA than the probe polynucleotide and has
two features of significance: (a) stable but reversible
binding to the probe at a specific locus and (b) a label
susceptible to detection, especially after displace-
ment. These features are discussed herein separately,
followed by consideration of the effect certain types of
labeling have on stability and displacement.

The pairing between the labeled polynucleotide and
the probe polynucleotide will generally occur over a
smaller number of bases than the pairing between the
target nucleotide sequence and the probe. In most
cases, the bases of the probe polynucleotide to which
the labeled polynucleotide specifically binds are a sub-

set of the bases of the probe later binding to the
target nucleotide sequence of the sample, and thus
represent a portion of what is called above the target
binding region of the probe.

The term labeled polynucleotide binding region
(LBR) is used herein to refer to that sequence of bases
in the probe to which the labeled polynucleotide is
bound in the complex. In preferred embodiments (as
illustrated by all Figures) the labeled polynucleotide
binding region is totally part of the target binding
region (see especially Figure 1A); in other embodiments
(see Figure 1G) only a portion (but preferably the major
portion) of the labeled polynucleotide binding region is
a part of the target binding region. Lengths of labeled
polynucleotide binding region outside the target
nucleotide binding region of the probe polynucleotide
region that are greater than about 15 bases are not
preferred because of the potential difficulty of
disassociating the labeled polynucleotide from the probe
once the only attachment is via base pairing in this
region. In some of the preferred embodiments, the
region of the probe polynucleotide to which the labeled
polynucleotide binds is a subset at or near one end of
the larger region of the probe polynucleotide to which
the target nucleotide sequence of the sample
subsequently binds. In some such embodiments, if the
probe is immobilized in the reagent complex, the
aforementioned one end of the larger region is at or
near an end of a linear probe polynucleotide (as illus-
trated in Figure 1A and discussed below; note, however,
that the other end of the TBR may also be used as an
LBR).

The present reference to a labeled polynucleotide
and a probe polynucleotide as distinct entities should
not be understood, however, to be a requirement that
they are linked solely by complementary base-pairing or
that there is necessarily only one labeled
polynucleotide attached to each probe polynucleotide or

that there is only one labeling moiety (tag) per labeled polynucleotide. Other forms of attachment of probe to labeled polynuclotide may also be present, but are generally not preferred. In embodiments without a separation, severing such other attachment of probe to labeled polynucleotide may or may not be required, depending upon whether or not displaced labeled polynucleotides still attached by such other means appear in the detection method as if they were totally displaced. Severing such other attachment before, during or after displacement but, in any event, before the determination step is preferred. Multiple labeled polynucleotides on a single probe strand (as illustrated in Figure 6) is a form of the invention which may have special application when greater numbers of displaced tags per displacement event are desired.

The size of the labeled polynucleotide binding region of the probe is not itself critical to the present invention, because wide variations in this size can be compensated for, e.g., by modifying the temperature of the displacement step (contacting step (b) described above) and by the size of the region of the probe to which the sample binds. A generally preferred size for labeled polynucleotide binding region (and the corresponding base sequence of the labeled polynucleotide) is at least about 15 bases, preferably at least 20 bases, and more preferably at least about 25 bases. A preferred range is about 20 to about 1000 bases (more preferably about 20 to about 500 bases, and most preferably about 25 to about 200 bases). Labeled polynucleotides with unusually short pairing segments (giving regard to factors such as GC base content) may dissociate from the probe in a non-specific manner if the temperature of the displacement step is too high (also giving consideration to factors such as salt concentration which affect melting temperatures). See D. Freifelder et al, _Biopolymers_, vol. 7, pp. 81-93 (1969). There is no essential advantage in unusually

long pairing segments (e.g., over 1000 bases). Such
long pairing segments are sometimes less preferred
because the overall target binding region of the probe
could then become longer and require necessarily longer
target nucleotide sequences for successful displacement
of the labeled polynucleotide. The binding portion of
the labeled polynucleotide may, if too long, no longer
easily be manufactured by certain techniques presently
available or that are best suited for small
polynucleotides: e.g., organic chemical synthesis of
the entire labeled polynucleotide. Organic chemical
synthesis is generally easier at present for labeled
polynucleotide binding regions of less than about 100,
and especially less than about 60; however, improvements
in such synthetic techniques could make longer sequences
easy to make as well.

The minimum length of labeled polynucleotide (and
also of labeled polynucleotide binding region) is
related primarily to reagent complex stability. Factors
other than length which affect this stability include GC
content (whose effect on melting temperature is well
known) and internal base pair mismatches. Melting
temperature is a useful way to establish an effective
length for sequences having one or more internal base
pair mismatches. As an example, a sequence of 30 bases
having one internal base pair mismatch (and thus only 29
exact pairs) may perform (at least with regard to
reagent complex stability) effectively like a shorter
exactly matched sequence; the degree of departure from
behavior as a 29 base pair sequence will depend upon the
position of any base pair mismatches and the base change
that has been made. Effective length can be expected to
differ depending upon whether the mismatched pair is
purine/purine, purine/pyrimidine or pyrimidine/pyrimi-
dine. Any effective length can be empirically
determined, however, by melting temperature experiments
in which a series of probe/labeled polynucleotide
complexes are subjected to various temperature regimes,

as illustrated in Example 8 of parent application
U.S.S.N. 607,885, and in articles such as R. L. Ornstein
and J. R. Fresco, Biopolymers, vol. 22, pp. 1979-2000
(1983). By determining a melting temperature of a
complex of probe with a labeled polynucleotide
containing base pair mismatches, and comparing that
value with melting temperatures of complexes with
slightly shorter lengths of pairing (but perfect base
pair matching within the paired region) on the same
probe polynucleotide, the effective pairing length of
the labeled polynucleotide with base pair mismatches can
be estimated and applied to the above preferred and more
preferred ranges. The above correlations based upon
melting points are intended, however, primarily as an
easy estimation tool. The actual degree of preference
for a given labeled polynucleotide binding region is
based both on how well the labeled polynucleotide
actually stays in the complex during storage of the
reagent or contact with non-homologous nucleic acids
under use conditions and on how well the labeled
polynucleotide is displaced by nucleic acids having the
appropriate target nucleotide sequence. While base pair
mismatches are permitted, they are in general not
preferred and, when present, generally comprise no more
than two fifths and preferably comprise no more than one
tenth of the region of pairing (e.g., a preferable
maximum is three mismatches and twenty-seven perfect
matches in a 30 base pair long region of labeled
polynucleotide/probe polynucleotide pairing).

The labeled polynucleotide may contain regions of
nucleotides, in addition to the pairing region, which do
not specifically bind to the probe polynucleotide. Such
regions may serve to link the pairing region to the
detectable tag, may themselves be tagged (e.g., by
radioactive labeling or by covalent attachment to an
indirect marker such as avidin, steptavidin or biotin)
or merely be present without any particular function.
The labeled polynucleotide may itself be linear or

circular and may be (but is preferably not) double
stranded in regions other than the pairing region. The
labeled polynucleotide is preferably not circular when
the probe polynucleotide is circular because of possible
topological constraints on hybridization of two circular
nucleic acid molecules and possible constraints on
hybridization with the target nucleotide sequence.

One or more detectable tags may be generally
located (using conventional techniques) at one or more
of several points along the labeled polynucleotide
(especially if the tag is a radionuclide or biotin or
the like), only at one end or only at one specific
internal location on the labeled polynucleotide (e.g.,
at a purine or pyrimidine base not involved in base
pairing to the probe polynucleotide). Provided that
there is at least one region of the labeled
polynucleotide unpaired in the reagent complex, the tag
is preferably present or concentrated on or within such
unpaired region. Directly detectable tags which may be
used include radionuclides (especially phosphorus-32,
sulfur-35, carbon-14 or iodine-125 labeled nucleotides),
fluorescent compounds (such as fluorescein or rhodamine
derivatives attached to the free end of a labeled
polynucleotide or to one or more of the unpaired bases
of a labeled polynucleotide) or moieties directly
detectable by other means (including being cleaved off)
such as the moiety nitrophenol detectable
colorimetrically or otherwise. Indirectly detectable
tags include those modifications that can serve as
antigenic determinants, affinity ligands, antigens or
antibodies recognizable through immunochemical or other
affinity reactions such as described in EPA 63,879, WO
83/02277 and EPA 97,373, referenced above and
exemplified by biotinated nucleotides present in or
added onto the labeled polynucleotide (such as by the
enzyme terminal deoxynucleotidyl transferase which will
add multiple nucleotides at the 3' end of the labeled
polynucleotide in the absence of a template strand).

Other indirect tags include enzymes attached to a
labeled polynucleotide (especially at a free end remote
from the region paired to the probe) whose presence can
be determined after displacement and separation steps of
the embodied method by addition of the substrate for the
enzyme and quantification of either the enzymatic sub-
strate or, preferably, the enzymatic reaction product.
Similarly, the tag may be an apoenzyme, co-enzyme,
enzymatic modifier, enzymatic cofactors or the like,
with the other necessary reagents usually added after
displacement (and, in certain embodiments, after
separation) along with the appropriate enzymatic
substrate, after displacement (and, in certain
embodiments, separation). Of course, if the enzymatic
reaction cannot occur with all but one component present
(e.g., the substrate), then these other reagents may be
present in solution during the contacting (displacement)
step (b) described above.

Multiple detectable tags can be added in
manufacturing the labeled polynucleotide such as by
using a terminal deoxynucleotidyl transferase enzyme.
Multiple labeled polynucleotides, e.g., one containing
the enzyme (or apoenzyme) and one containing the
coenzyme, can also be used. One form of attachment of
an enzyme to the labeled polynucleotide is via affinity
reagents, e.g., streptavidin to biotin. Such a binding
form can be used in various embodiments, for example
wherein the complex is prepared by hybridizing a biotin-
labeled polynucleotide to the probe and then binding a
streptavidin-enzyme conjugate to the biotin prior to the
contacting (displacement) step (b), described above.
Furthermore, a moiety interacting with the detectable
tag in the complex may be present on the probe.

Most forms of detectable tags, especially if remote
from the pairing region of the labeled polynucleotide to
the probe, will have little or no effect on the strength
of base pairing between the labeled polynucleotide and
the probe polynucleotide, as evidenced (for testing

purposes) by little or no diminution of the reagent
complex melting temperature and, more importantly, by
negligible effects on the hybridization reaction between
any target nucleotide sequence and the probe polynucleo-
tide. Some forms of labeling, such as covalently bound
biotin on nucleotides of the labeled polynucleotide in
the base pairing region and such as a large enzyme
molecule or fluorescent moiety linked to nucleotides in
or near the pairing region, may have an appreciable
effect on reagent complex stability. Such effect
generally will be to destabilize the labeled
polynucleotide/probe polynucleotide binding (and thus
lower its melting temperature). That effect may be
somewhat beneficial in speeding up displacement, but can
cause increases in non-specific dissociation or "fall-
off" of the labeled polynucleotide. Such non-specific
"fall-off" can usually be reduced, however, by lowering
the temperature during the displacement step, increasing
the length of the labeled polynucleotide binding region,
or other such modification of the physiochemical
properties of the system.

Forming a reagent complex between an immobilized
probe polynucleotide and a labeled polynucleotide (such
complex being provided in the present process and being
in the present reagent) may involve attachment of the
probe to a support (as described above) either before or
after hybridization of the labeled polynucleotide to the
probe polynucleotide. Affinity or other chemical
reagents may be used for mediating or participating in
such attachment. If the immobilization is completed
after hybridization of the labeled polynucleotide to the
probe polynucleotide, then the linking moiety or a part
thereof can already be attached to the probe.
Generally, the formation of such an immobilized complex
will be followed by washing off unbound labeled nucleo-
tide, and the conditions of such washing may be designed
to also remove labeled polynucleotides that are only
slightly bound to the probe (e.g., through less than

about fifteen complementary bases elsewhere on the
probe, instead of through the larger number of
complementary bases at the desired binding site) or are
adsorbed to the support. Probe polynucleotides and
complexes of probe polynucleotide with labeled poly-
nucleotide that are only marginally attached to the
support may also be removed during this washing step.
The washing should preferably be under sufficient
conditions and for a sufficient time to substantially
eliminate the non-specific background signal due to
labeled polynucleotides (with or without probe poly-
nucleotide) separating from the support independently of
specific displacement during the displacement step of
the present method. One can also use a reagent (e.g.,
protein) to block any non-specific binding sites on the
support.

In the manufacture of reagent complexes which are
in solution when used, it is frequently also desirable
to separate labeled polynucleotides which have not bound
to probe polynucleotides from product reagent complexes
(in some instances unbound probe polynucleotides may
also be removed). Such separation may be by size alone
(e.g., by size exclusion chromatography) if, as is
frequently the case, the labeled polynucleotide is much
smaller than either probe polynucleotides or reagent
complexes. Such separation may also be based upon the
double-stranded nature of at least one portion of the
reagent complex (at the labeled polynucleotide binding
region of the probe) where such a double-stranded region
is not likely to be present in either the labeled
polynucleotide or probe polynucleotide, both of which
would be expected to be in single-stranded form except
for very small internal binding regions. This property
renders reagent complexes separable from unbound labeled
polynucleotides by, e.g., affinity chromatography using
double-strand specific anti-nucleic acid antibodies or
hydroxylapatite chromatography.

In some instances, the labeled polynucleotide and

probe polynucleotide can be part of the same polynucleo-
tide chain. For example, a linear single-stranded DNA
molecule can be constructed from an M13 bacteriophage
which contains a cloned DNA insert with inverted
repeats. These inverted repeats, which are capable of
forming a double-stranded region due to their
complementarity, would include the labeled
polynucleotide and the labeled polynucleotide binding
region. Sequences located between or adjacant to these
repeats would include the initial binding region,
located adjacent to the labeled polynucleotide binding
region. Unique restriction enzyme cleavage sites, e.g.,
the M13mp7 polylinkers or modifications thereof, located
outside of the inverted repeats and the initial binding
region, could be cleaved to release the cloned insert
from the single-stranded M13 vector backbone (c.f. G.A.
Ricca, J.M. Taylor & J.E. Kalinyak, Proc. Nat. Acad.
Sci. U.S.A., vol. 79, pp. 724-728 (1982). An additional
small inverted repeat sequence, containing a restriction
enzyme cleavage site (e.g., the M13mp7 polylinker) could
be placed between the labeled polynucleotide repeats.
Cleavage at such a site (e.g., X of Figure 3D) would
leave only complementary purine/pyrimidine base pairing
to hold the labeled polynucleotide attached to the now
distinct probe polynucleotide and would provide free
ends on the probe polynucleotide through which
attachment to a solid support could be mediated if so
desired. Any tag or tags may be added to the labeled
polynucleotide at this point if not already present.

     If the volume-excluding polymer used in some forms
of the present invention is a polyether, it may be a
simple poly(alkylene oxide) such as those of the formula
$H(O-R)_n-H$ where R is alkylene of 1-6 carbons and n is an
integer (on a weight average basis) of 15 - 1000. R can
be methylene, 1,1-ethylene, 1,2-ethylene, 1,2-propylene
1,4-butylene, 1,2-butylene, 1,2-hexene (2-butylethene)
or similar divalent saturated hydrocarbon moieties, and
can be mixed within a single molecule. Preferably R is

1,2-ethylene or 1,2-propylene as in poly(ethylene glycol) (PEG, also called poly(ethylene oxide) or PEO) or as in poly(propylene glycol) (PPG, also called poly(propylene oxide) or PPO). The integer n in such instances represents the weight average molecular weight divided by the monomeric unit molecular weight; such monomer unit molecular weight for PEG is 44 and for PPG is 56. For PEG, weight average molecular weights are preferred empirically to be at least about 1500, preferably at least about 2500 and more preferably between about 3000 and about 20,000. Accordingly, n (on a weight average basis) is preferably at least 36, more preferably at least 60 and most preferably between about 70 and about 690. A similar set of preferred ranges for n in PPG is expected.

Also included as suitable polyether polymeric agents in the present invention are condensation products of ethylene oxide, propylene oxide or other alkylene oxides on various moieties such as diols, triols, sugars or acids. Such materials are well known in the art of non-ionic detergents and may be effective in the present invention provided that the molecular weight (and water solubility) is sufficient to enhance displacement without unduly enhancing non-specific dissociation of labeled polynucleotide from the probe polynucleotide.

In certain broader forms of the invention, however, other chemically inert, water-soluble nonionic or anionic polymers may be used as the volume excluding polymer in place of or in conjunction with the above polyethers. Examples include polyacrylates, poly(vinyl acrylates), derivatized and underivatized polysaccharides, polyphosphoric acid, and various copolymers containing pendant carboxyl, sulfonate, sulfate, phosphate, or other anionic moities. A material shown to have limited beneficial effects is FICOLL, MW 400,000 (a cross-linked reaction product of sucrose with epichlorohydrin having free hydroxyls;

FICOLL is Pharmacia's trademark). A class of suitable non-ionic volume excluding polymers is thus the reaction products of a sugar with epichlorohydrin.

Certain volume excluding polymers are preferably used in the total method of the invention, involving both the contacting (displacement) step and the determining (detecting) step only in forms in which their beneficial effects upon the displacement reaction are not outweighed by deleterious effects upon the determination step or a portion of the determination step. For example, in those embodiments wherein the determination step includes a separation (e.g., gel chromatography, size exclusion chromatography, affinity chromatography or electrophoresis), the presence of certain volume excluding polymers may have deleterious effects. Dextran sulfate, while believed to be useful in promoting displacement, was observed in several experiments to have deleterious effects upon electrophoresis (under the particular conditions tested) and in more limited experiments to have deleterious effects upon size exclusion chromatography (under the particular conditions tested). It is believed, however, that one can find, through routine experimentation, suitable polymers for any particular embodiment of the present method and can find, through routine experimentation, suitable detecting or determining conditions for using any polymer which has the effect of promoting the hybridization and displacement process.

The actual contacting or displacement step with sample material (potentially containing nucleic acids that may include the target nucleotide sequence) will normally be under conditions of temperature, ionic strength, polyether polymer (or similar volume-excluding polymer) concentration and time less stringent (and thus less conducive to non-specific uncoupling of the labeled polynucleotide) than the above washing step. A desirable temperature range during the contacting step

is from about 15°C to about 90°C, depending upon the
solution ionic strength and other additives affecting
melting temperature; the most efficient temperature will
be one at which a maximum or near maximum rate of
hybridization of sample nucleic acids to probe occurs.
In certain cases, however, a more convenient (generally
lower) temperature such as from near ambient temperature
(15-25°C) up to physiological temperatures (37-40°C) may
be used. As described in a number of literature
references (e.g., J.G. Wetmur and N. Davidson, J. Mol.
Biol., vol. 31, pp. 349-370 (1968) and C. Minson and G.
Darby, New Developments In Practical Virology, vol. 1,
pp. 185-229 (Alan Liss, Inc., N.Y., 1982)),
hybridization rate is also a function of pH and sample
nucleotide concentration. Also, in addition to the
application of a polyether polymer in the preferred
assay methods described herein, other water soluble
volume excluding polymers such as dextran sulfate can be
used in conjunction with the polyether polymer. Enzymes
and other proteins which affect the displacement process
(such as the E. coli ATP-dependent rec A protein) may
further be present, however, based upon their beneficial
effects described in our copending, commonly assigned
application (whose priority documents are U.S.S.N.
607,885 and 684,305) filed herewith.

Proportions, amounts and concentrations of reagent
complex and, if used, polyether polymer are not
independently critical, but it is generally desired that
the total hybridization mixture of sample and reagent
complex be as concentrated as feasible. In most
instances, probe polynucleotides bearing binding sites
for the target nucleotide sequence will be expected to
be present in ten-fold or more molar excess (possibly
hundred-fold or more molar excess) of any anticipated
level of target nucleotide sequence in the sample. The
sample itself may include nucleic acids which preferably
should be completely or partly in solution (separated
from membranes and the like) and in single-stranded form

for the hybridization step of the assay. The presence of the unlabeled complement of the target nucleotide sequence (by virtue of denaturation of double-stranded sample DNA) could represent an interference. This interference is likely to be minor in at least the preferred forms of the invention due to the usual molar excess of reagent over sample strands having the target nucleotide sequence or its complement; in hybridizations involving immobilization of the probe selectively (before or after displacement), displaced labeled polynucleotide will be and will remain in the solution phase and be subsequently determined, whether or not such displaced labeled polynucleotide has rehybridized with complementary segments of the sample nucleic acid. In many solution hybridizations this interference may also be minimized by kinetic effects.

In general, the optimal proportions of polyether polymer, if used, can be expressed as a weight proportion (weight/vol or weight %) of the total aqueous phase during the displacement reaction. Such proportion will generally be from 2% up to the solubility limits of the polyether polymer and will preferably be between about 5% and about 20% (weight %). For particular reagent complex and displacement conditions, optimal proportions and appropriate polymer molecular weight can be further determined by routine experimentation as illustrated in Examples 13 and 14.

The basis for the volume exclusion phenomenon, incompatability of two water soluble polymers (in the present case being the nucleic acid and the volume excluding non-ionic or anionic polymer), is summarized in P. Molyneux, Water-soluble Synthetic Polymers: Properties And Behavior, Vol. 2, pp 167-170 (1984) (see also references cited therein).

In general, displacement reactions using volume excluding polymers should require no more than three hours, generally under one hour and desirably less than thirty minutes to occur to a sufficient extent for

detection. Conditions often can be adjusted to achieve substantial completion of strand displacement reactions within these times. Longer incubation times are not necessarily disadvantageous, however. It is believed that the rate-limiting step is a sample or target nucleotide sequence finding a complementary sequence of the probe polynucleotide (cf. C. Green and C. Tibbetts, Nucleic Acids Research, vol. 9, pp. 1905-18 (1981)); once target/probe hybridization begins to occur, displacement of labeled polynucleotide is expected to occur from each individual reagent complex in under one minute and frequently less than one second.

In some of the forms of the invention in which the complex is initially on a solid support, the liquid phase containing displaced labeled polynucleotide may be separated therefrom as a part of the determination step. If the complex is in solution, some forms of the invention involve treatments after the contacting (displacement) step to fix reagent complexes still present (and, unavoidably, in some cases other forms of the probe including those hybridized to a target nucleotide sequence) to a solid support, followed by a similar solid/liquid separation. Such separation of the solid phase containing bound complex from liquid phase containing displaced labeled polynucleotide may be by physical means such as chromatography, filtration, centrifugation, magnetic attraction or decantation. The solid phase may include magnetic or other separable particles that are attracted or otherwise physically removable from the liquid phase. Furthermore, complete separation of the two phases is not required; an aliquot of the liquid phase following partial separation may be removed for label determination, leaving the solid phase admixed with the remainder of the liquid phase. If such a separation occurs or is used, determination (detecting) of the presence and frequently the quantity of labeled polynucleotide present may be conducted upon either phase, but is preferably conducted upon the

liquid phase. Determination of the label in the liquid phase as a measure of the presence and quantity of displaced labeled polynucleotide typically has the advantage of generally lower background signals. Any background signal will be largely that caused by non-specific fall-off from a solid support as described above, by non-specific dissociation of labeled polynucleotide strands from reagent complexes, or by imperfect preparation of reagents. Additionally, the absence of target nucleotide sequence in the sample results in the proportional absence of signal above background levels from labeled polynucleotide in the liquid phase. By contrast, if detection is of the label associated with the solid phase, a negative result is indicated by unreduced levels of labeled polynucleotide. Especially when a high molar excess of reagent complex over sample nucleotide sequences over anticipated target nucleotide sequences is used, measurement of the label remaining on the solid support results in substantially reduced sensitivity.

In some embodiments of the invention, no post-displacement separation occurs, and determination can still be made of displaced labeled polynucleotides. Some such determination (detecting) steps involve a change in the signal detectable from a tag by virtue of displacement, including those signals affected by proximity of the tag to a signal-affecting moiety elsewhere on the labeled polynucleotide or on the probe polynucleotide (see description of Figure 3A-3D, below).

In other forms, especially with immobilized probes, the interaction can be between two types of tag moieties. One type of detectable tag can be on labeled polynucleotides hybridized to immobilized probe polynucleotides at one location on a solid support. A second type of tag may be on labeled polynucleotides which are hybridized to immobilized probe polynucleotides at a remote location on the same solid support or on a separate solid support in close physical

proximity. The second type of tag may also be otherwise directly attached to some remote location of the probe or of the same labeled polynucleotide or of the solid support. In all such cases involving two types of tags, the two different tags can interact only if at least one labeled polynucleotide (tag) is displaced. Such interaction is especially applicable to apoenzyme with coenzyme: e.g., apoglucose oxidase with flavin adenine dinucleotide (FAD).

The process and reagent of the present invention can be used for the detection and determination of a variety of target nucleotide sequences in a variety of concentrations. In particular, microorganisms including infectious agents whose nucleic acid (genomic or other-wise) can be targeted include pathogenic viruses, bac-teria and fungi; e.g., cytomegalovirus or Neisseria gonorrhea. Exemplary genetic disorders or conditions which can be targeted include β -thalassemias, α_1-thalassemias, cri du chat syndrome and some retino-blastomas. The present process and reagents are applicable to detecting genetic disorders or variations primarily when a multi-base nucleotide deletion, insertion, substitution or transposition is involved in distinguishing the target nucleotide sequence from the closest sequence present in samples intended to be read as negative for the target sequence. To the extent that the present invention is applicable to genetic disorders due to single base mutations, if at all, the complement of the substituted base or other point of mutation is desirably part of the target binding region of the probe polynucleotide, with the location of that base within the region likely to affect the selectivity of the method. Changes or differences in the expression, activation or rearrangement of structural genes, regulatory genes or oncogenes can be detected by the present method. For monitoring gene expression, mRNA would be targeted. Other perturbations in the expression of structural genes can be similarly

detected.   The present process

can also be applied to histocompatability typing for
tissue transplantation, determination of antibody
resistance genes in microorganisms, and to the screening
of food, medicinal and water samples for specific
infectious agents or other microorganisms.

Selecting a target sequence for a particular test
involves determining a sequence which is unique or
relatively unique to the target organism or condition.
Such target sequence would be used to develop the target
binding region (which is complementary thereto) and then
a labeled polynucleotide of appropriate length would be
developed to bind to a part of the target binding
region. In some instances multiple reagent complexes
targeting different parts of the nucleic acid of the
same organism or condition may be used in order to
impart improved specificity when any particular target
sequence is only relatively unique.

One embodiment of the present invention is illus-
trated, for purposes of understanding, by reference to
attached Figures 1A-1E, in which is shown an immobilized
probe polynucleotide P, 3300 nucleotides in length.
Numbering from the 5' end, the region from nucleotide
3000 to nucleotide 3300 represents the target nucleotide
binding region (TBR). For simplicity, it is assumed
that the first nucleotide of the probe is directly
attached to a support S. As illustrated in Figure 1A,
the labeled polynucleotide L consists of 150 bases to
which a tag T is attached at the 5' end. Of these 150
bases, 51 (from base number 100 to base number 150) bind
specifically to the labeled polynucleotide binding
region (LBR), bases 3250 to 3300, of the probe. Accord-
ingly, when the immobilized probe consisting of probe
polynucleotide P attached to support S is incubated with
a solution of labeled polynucleotide L under normal
hybridization conditions, a complex will be formed as
shown in Figure 1A with labeled polynucleotide L
attached at the end of the probe polynucleotide P via
base pairing between bases 3250-3300 of the probe P and

bases 100-150 of the labeled polynucleotide L.

In use, the illustrative complex is contacted with a biological sample under hybridization conditions where the sample contains a nucleotide sequence with bases 10000 to 10300 of this sequence which corresponds to and binds selectively to the target binding region TBR of the probe. Under proper hybridization conditions, the sample polynucleotide G would first bind (form base pairs), as illustrated in Fig. 1B, from base 10051 to base 10300 of G and base 3000 to base 3249 (the initial binding region or IBR) of probe P. The IBR in the reagent complex shown in Fig. 1A is entirely single stranded. It should be appreciated that thermodynamic considerations favor the formation of double-stranded moieties such as shown in Fig. 1B over single-stranded moieties. It is believed, however, that this initial hybridization is the rate-limiting step and that the present use of volume excluding polymers facilitates or speeds up this step under many conditions. Thereafter, an equilibrium is created at bases 3250 to 3300 (LBR) of the probe polynucleotide P between binding to the labeled polynucleotide L and binding to bases 10000 to 10050 of the sample polynucleotide G. Under these conditions, a rapid zippering and unzippering will occur, as represented by the differences between Figure 1B on the one hand and Figures 1C on the other hand, along with random migration of the branch point along the probe polynucleotide. In such event, when additional bases of the sample polynucleotide G bind to the probe polynucleotide beyond probe base 3249, corresponding bases of the labeled polynucleotide L are displaced, creating a free end. Under the conditions described herein, this zippering and unzippering can occur in either direction; but the favored event will be eventual shifting of the point of attachment to the right in Figure 1C. Figure 1D shows the stage of near total labeled polynucleotide strand displacement in somewhat enlarged and more graphic form from base number

3275 on the probe P toward base number 3300, at which
point the labeled polynucleotide L is totally displaced
from the probe P (cf. Figure 1E). It should be appre-
ciated that, merely upon random zippering and unzip-
pering in both directions, removal of the labelled
polynucleotide L will be favored in that Figure 1B
represents the furthest to the left that the labeled
polynucleotide L can displace the target nucleotide
sequence of sample polynucleotide G. As long as some
substantial region of the initial binding region IBR
exists in which a sequence of nucleotides of the probe
polynucleotide P binds selectively to the target nucleo-
tide sequence, but not to the labeled polynucleotide,
displacement of the sample polynucleotide G completely
from the probe will be a rare and reversible event.

An important parameter in optimizing the present
method and reagent is the length and nature of the base
pairing between the labeled polynucleotide and the probe
polynucleotide. As compared to the 51 bases of exact
pairing illustrated in Figure 1A, modification may be
made either by shortening or increasing this length, by
introducing mismatches or loops in either strand or by
selecting between DNA and RNA for either or both strands
so as to affect the rate of spontaneous (and presumably
also specific) dissociation. In this regard, certain
differences can be achieved when the target nucleotide
sequence is RNA and the labeled polynucleotide is DNA,
whether the probe polynucleotide P is either DNA or
RNA. This is because, in general, RNA-RNA binding is
strongest per base pair, DNA-RNA base pairing is of
intermediate strength and DNA-DNA base pairing is of the
least strength per base pair. If, however, the target
binding region (TBR) is larger than the labeled
polynucleotide binding region (LBR), then the labeled
polynucleotide can be RNA and still be displaced by a
DNA target nucleotide sequence. Targeting RNA may be
required if, for example, the target microorganism is an
RNA virus or the target condition is one of altered gene

expression.

One means of reducing the binding strength in the region in which the labeled polynucleotide is bound to the probe polynucleotide is the introduction of individual base mismatches into the labeled polynucleotide. Assuming that the probe nucleotide sequence will be chosen to pair exactly or nearly exactly to the target nucleotide sequence, such mismatches can be considered as mutations or individual base substitutions in the labeled polynucleotide compared to a similar polynucleotide segment of the target nucleotide sequence which has exact (or nearly exact) binding. Such mutation or substitution may include the substitution of a natural or chemically modified nucleotide for a given natural nucleotide such as the following: G for A (to produce the apposite pair dG-rU, dG-dT, rG-dT or rG-rU), A for G, 5-methylcytosine for C, 5-hydroxymethylcytosine for C, gentibiosyl-5-hydroxymethylcytosine for C, or 5-bromouracil for A. In many preferred embodiments, such mutations involve the substitution of one naturally occurring nucleotide for another nucleotide. In certain such embodiments, the substitution involves the substitution of a pyrimidine for a purine, leading to a mismatched pair that has become a pyrimidine-pyrimidine pair. Because pyrimidines occupy less space than purines, such individual pyrimidine-pyrimidine mismatches can have a minimal effect upon adjacent nucleotide pairings. Purine-pyrimidine mispairings (for example, A being positioned opposite to C, or T or U being positioned opposite to G) are somewhat more space filling, but are still generally less space filling than the positioning of two purines (A-A, A-G or G-G) apposite each other. Counteracting the space-filling effect, however, is a stacking energy effect which runs in the opposite direction: generally, purine/purine base pairs display a lower free energy of stacking, purine/pyrimidine base pairs somewhat more and pyrimidine/pyrimidine base pairs somewhat more still. See R. L. Ornstein & J.

R. Fresco, Biopolymers, Vol. 22, pp. 1979-2016 (1983)
(two articles). The net effect, in terms of effect on
melting temperature of the reagent complex, in terms of
effect on stability of the reagent complex and in terms
of effect on the displacement kinetics, will primarily
represent a combination of these two counteracting
effects, such that experimentation may be required to
determine which, if any, mismatches may be preferably
incorporated into the labeled polynucleotide for a par-
ticular probe after the position of the target nucleo-
tide region and labeled polynucleotide region are fixed.

In addition to point substitutions, mismatches can
be created either by inserting a short sequence in the
labeled polynucleotide L which does not correspond to
the probe nucleotide sequence (e.g., inserting a series
of A's between bases 125 and 126 of labeled polynucleo-
tide L in the example illustrated in Figure 1A) or by
deleting a portion (such as by deleting bases 120-130 of
labeled polynucleotide L in Figure 1A). Insertions in
the labeled polynucleotide will generally form a single-
stranded loop or cruciform structure of the labeled
polynucleotide; deletions therein will generally form a
single-stranded loop or cruciform structure of the probe
polynucleotide.

Such substitutions, deletions or insertions will
have the effect of destabilizing the binding between the
labeled polynucleotide L and the probe polynucleotide P
such that the displacement of the labeled polynucleotide
may be increasingly favored. It is important, however,
to avoid to the extent possible nonspecific displacement
of the labeled polynucleotide L from the probe
polynucleotide P in the absence of the target nucleotide
sequence. The minimum length of binding between the
labeled polynucleotide L and the probe polynucleotide P
which is sufficient to minimize such dissociation or
falling off will be dependent on a number of factors
including, especially, the conditions such as pH and
temperature of hybridization, the mode of attachment of

the probe polynucleotide to the support (e.g., end attachment versus nonspecific adsorption), the extent of destabilizing substitutions (or deletions or additions), the duplex base sequence at the region of hydrogen bonding and whether the binding is between RNA and RNA, DNA and RNA or DNA and DNA. The optimal conditions in a particular instance can be determined empirically with routine experimentation based upon the general teachings of the present disclosure. Such experimentation may involve melting temperature experiments with samples lacking the target nucleotide sequence for purposes of estimating stabilities, and then displacement experiments for final optimization.

In considering the geometric relationship between the region where the labeled polynucleotide binds to the probe polynucleotide (LBR) and the region where the target nucleotide sequence binds to the probe polynucleotide (TBR) a configuration such as that illustrated in Figures 1A-1B may be used, with the labeled polynucleotide binding region being a subset of and at the end of the target binding region distal to the solid support. A common end of the TBR and the LBR (probe nucleotide 3300 in Figure 1A) may also be (or be near to) one end of the probe polynucleotide. There are, however, no great disadvantages in having the probe polynucleotide extend beyond this point in a sequence of a nonspecific nature. There may be situations in which it is desirable to have a probe polynucleotide continue beyond this pairing region and extend to a point of attachment of a tag (different from the tag T on labeled polynucleotide L) which is to be released subsequently by other techniques. Furthermore, the two tags (one on the labeled polynucleotide, the other in the probe polynucleotide) may interact, with the interaction being detected as a part of the read-out. It may be preferred in certain embodiments of the invention that the labeled binding region be near or at the end of the target binding region nearest the support.

Situations which are included in the present inven-
tion, but which may be less preferred include extension
of probe polynucleotide P beyond the LBR (that is, for
example, for the 125 bases beyond base 3300 in Figure
1F) with a region (such as bases 3375-3425) which binds
selectively to a portion of the sample nucleotide (and
especially a region such as bases 9750-9800 of the sam-
ple polynucleotide G). In such an event, the sample
polynucleotide G could bind both at bases 3000-3249 of
the probe polynucleotide P and at bases 3450-3500 of the
probe polynucleotide P and overlay the labeled poly-
nucleotide L. Such a structure, represented generally
as a "triple-stranded" region or D loop (see C. Green
and C. Tibbetts, Nucleic Acids Research, vol. 9, no. 8,
pp. 1905-18 (1981), especially page 1912), might not
cause displacement of the labeled polynucleotide L in
certain topologies and thus would represent a potential
specific binding event without a signal generation.
While displacement at bases 3250-3300 of the probe might
still be possible (and might even proceed from both
ends), the loss of free movement of the sample strand G
due to binding at its bases 9750-9800 may reduce the
probability of complete displacement, especially when
one considers the helical structures actually involved.

Another situation within the scope of the present
invention, but also somewhat less preferred than the
above, is that in which the labeled polynucleotide bind-
ing region (LBR) extends beyond and outside the target
binding region (TBR). This second embodiment of the
invention is illustrated in Figure 1G (and in Examples
13 and 14), wherein the reagent complex is shown, with
the target nucleotide sequence being capable of displac-
ing the labeled polynucleotide as far as the end of the
TBR. The labeled polynucleotide would then remain bound
via those probe bases which are part of the labeled
polynucleotide binding region LBR but not part of the
target binding region TBR (such sequence of bases being
designated the residual binding region or RBR). Pro-

vided that the RBR (e.g., bases 3300 to 3305 of probe P
in Figure 1G) is much smaller than the LBR, one could,
in theory, change conditions at this point (pH or tem-
perature for example) so as to totally release the
labeled polynucleotide L in this complex without releas-
ing other labeled polynucleotides bound to the entire
labeled polynucleotide sequence LBR (see Example 13
where this is described for a displacement in solu-
tion). One should consider, however, that the hybrid
shown in Figure 1G will still be capable of displacement
to the left after complete binding of a target nucleo-
tide sequence G, with the labeled polynucleotide capable
of displacing part or all of the portion of the target
polynucleotide sequence bound to bases within the over-
lap of TBR and LBR; such an event could be expected to
lower the efficiency of displacing the labeled poly-
nucleotide.

Other embodiments of the invention different from
those illustrated in Figures 1A through 1G are illus-
trated in Figures 2-6 and discussed briefly below.

Figure 2 illustrates a third embodiment of the
invention using a complex different from that of Figure
1A in that the labeled polynucleotide L_1 is the circular
plus (or infectious) strand ($M13^+$) of DNA bacteriophage
M13 into which is inserted a segment A_1' complementary
to a portion (LBR) of the target binding region A of the
probe strands P_1 and P_2. The label in this embodiment
is shown as a series of biotin moieties B distributed
within the $M13^+$ major portion of the labeled polynucleo-
tide L_1, possibly by growing M13 in bacterial culture
with biotinated nucleotide triphosphate (e.g. biotinated
- dATP) present or by chemically attaching biotin after-
wards to the M13 nucleotides. The A'_1 segment may also
contain nucleotides bound to biotin. The sample polynu-
cleotide G will, in this instance (Figure 2), contain a
target nucleotide sequence A' complementary to segment A
of each probe P_1 and P_2. In this embodiment the probe
polynucleotides may also be formed using bacteriophage

M13, with segment A complementary to the target nucleo-
tide sequence A' inserted at a location in the non-
essential region of the M13 genome. The $M13^+$ segments
of the probe are non-complementary (actually equal or
homologous) to $M13^+$ regions of the labeled polynucleo-
tide. If the circular phage is cleaved non-specifically
(e.g., by partial cleavage with restriction enzyme Hae
III; c.f. R.W. Blakesley and R.D. Wells, Nature, vol.
257, pp. 421-422 (1975)), linear strands will be created
having segment A positioned at different points relative
to the ends, but generally with a portion of the $M13^+$
strand on both sides of segment A. This is illustrated
in Figure 2 by strand P_1, wherein the segment A is close
to the free end of supported polynucleotide, and strand
P_2, wherein segment A is close to the attached end. Of
course, cleavage will also potentially produce shortened
strands (due to two or more cleavages in the $M13^+$
region) and could also produce strands with region A
split between the two ends (due to cleavage in region
A); the latter condition can result in failure to form a
complete and fully operative target binding region. So
long as region A is small relative to the length of the
$M13^+$ strand (7.2 kilobases; J. Viera & J. Messing, Gene,
vol. 19, 259-68 (1982)), relatively few probe strands
would be expected to have region A split. Uniformity in
location of segment A can be achieved by cleaving
single-strand M13 DNA specifically (e.g., with a
restriction enzyme recognizing an inverted repeat
sequence in or inserted into the M13 genome).

After displacement, the biotinated displaced
labeled polynucleotides of Figure 2 or related embodi-
ments could be concentrated from the liquid phase, e.g.,
with a strepavidin column, before readout using, e.g.,
horseradish peroxidase linked to strepavidin and colori-
metric, fluorimetric or chemiluminescent readouts based
upon the peroxidase activity.

Figure 3A illustrates a fourth embodiment of the
present invention with the reagent complex entirely in

solution (no support is present). The probe polynucleo-
tide P has a target binding region (TBR) including a
subregion, the labeled polynucleotide binding region
(LBR), in which bases are bound to complementary bases
of the labeled polynucleotide (L). The labeled poly-
nucleotide (L) contains a fluorescent tag (F) held in
the complex in close proximity to a quencher moiety (Q)
attached to the probe (P). These moieties F and Q are
sufficiently close in the complex of Figure 3A for any
signal produced by stimulation of the fluorescent tag
(F) by radiation to be absorbed by the quencher moiety
(Q). An exemplary pair of F and Q are fluoroscein with
rhodamine. See M. Cobb and S. Gotcher, Am. J. Med.
Tech., Vol. 48, No. 8, pp. 671-677 (1982).

Figure 3B illustrates the reagent complex of Figure
3A after contact by a sample G containing the base
sequence complementary to the entire target binding
sequence (TBR). As in Figure 1B, binding will be first
to the unpaired region of TBR and then displacing por-
tions of the labeled polynucleotide L from the subregion
LBR. The stage of partial displacement (such as seen in
Figures 1C and 1D) is illustrated in Figure 3B. Upon
completion of the displacement, the target sequence of
sample polynucleotide G will be bound to the entire
region TBR of probe P and the labeled polynucleotide L
will be totally displaced. In this situation, as illus-
trated in Figure 3C, the tag (F) is now sufficiently far
from quencher moiety (Q) to produce a detectable signal
on stimulation that is not quenched (except in those
rare cases where another probe P happens to be located
such that its label Q is in close proximity to tag F).
Accordingly, the stimulation of fluorescent tag (F) and
measurement of unquenched signals can serve as a quan-
titative detection method for displaced labeled poly-
nucleotides without a separation step having been per-
formed.

A modification in the embodiment of Figures 3A-3C
in which the labeled polynucleotide L and probe polynu-

cleotide P (bearing, respectively, labels F and Q) are
part of the same molecule is illustrated in Figure 3D.
In addition, a site X is shown which contains an
inverted repeat sequence a portion of which is recog-
nizable by a restriction enzyme. Cleavage by such an
enzyme can be used to sever the covalent linkage between
TBR and L (although this severing is not required when
the read-out is based upon the F-Q interaction). Such
cleavage can also be used as a first step in creating a
site for attachment of the probe to a support, such
attachment to occur before or after displacement. The
overall geometry of Figure 3D may thus be used in syn-
thesizing reagent complexes as a single molecule for
various embodiments, including those of Figures 1A-1E.

Figure 4 illustrates a complex in which the probe
strand P_3 is a circular single-stranded polynucleotide
non-specifically adsorbed on support S. Such a poly-
nucleotide can be produced by insertion of a target
binding region (TBR), into a single-stranded bacterio-
phage such as M13 through suitable cloning procedures.
The labeled polynucleotide L here contains a segment A'_1
complementary only to a portion LBR of segment TBR.

Figure 5 illustrates a complex similar to Figure 4
except that, instead of being adsorbed to support S,
probe strand P_4 is linked via a linking polynucleotide
covalently attached to support S. Such linking polynu-
cleotide contains a region H complementary to a segment
H' of the probe P_4 remote from the TBR segment.

Figure 6 illustrates a complex similar to the
embodiment of Figure 1A in that the probe strand P_5
contains a target binding region TBR near or at its free
end. A number of portions of the TBR segment (subseg-
ments A_3, A_4, A_5, A_6, A_7, A_8, A_9, and A_{10}) are each base
paired to a complementary region of a labeled poly-
nucleotide: segment A'_3 of labeled polynucleotide L_3 to
subsegment A_3, etc. Probe P_5 contains a target binding
region TBR divided into subsegments A_1 and A_2, together
forming the initial binding region, as well as eight

additional subsegments (A_3 thru A_{10}), each forming a labeled polynucleotide binding region. In this embodiment, binding of segment A' of the sample polynucleotide G (as shown in Figure lE) will displace all of the labeled polynucleotides L_3, L_4, L_5, L_6, L_7, L_8, L_9 and L_{10} and thus produce an enhanced signal compared to the situation shown in Figure lA. It should be appreciated, however, that the absence of any of those labeled poly-nucleotide (e.g., L_8) could permit the target nucleotide to form D-loops (as in Figure lF) by binding to subsegments A_1 and A_2 (for which no labeled polynucleotide is complimentary) and any other probe subsegment (e.g., A_8) for which the labeled polynucleotide is absent on that particular probe strand.

EXAMPLES

In certain of the following examples, four small synthetic DNA oligomers were used as the tagged polynucleotides; all were labeled with radioactive phosphorous-32 atoms. Two oligomers were 27 base oligomers matching 26 or 27 bases at the 3' end of one strand of a 1.1 kb pBR322 DNA fragment. This fragment stretched from the Pst I restriction site to the Bam HI restriction site in pBR322 DNA. These two oligomers (L1 and L2), and the labeled polynucleotide binding region to which they were bound, are illustrated in Table 1. The mismatched pyrimidine base in oligomer L2 is underlined.

-45-

TABLE 1

350

Probe 5' end ...   T - A - C - G - C - G - A - T - G - A -

L1   3' C - T - A - G - T -

L2   3' C - T - A - G - T -

---

                        360   367

Probe T - G - G - C - G - A - C - C - A - C - A - C - C - C -

L1   A - C - C - G - C - T - G - G - T - G - T - G - G - G -

L2   A - C - C - G - C - T - G - G - T - G - T - G - G - C̲ -

       20   10   9

---

       370   380

Probe G - T - C - C - T - G - T - G - G - A - T - C - C . . .3'end

L1   C - A - G - G - A - C - A - C 5' end

L2   C - A - G - G - A - C - A - C 5' end

The two oligomers used in other examples were 32
base oligomers matching a region near the middle of a
1.1 kb human albumin gene sequence. The sequences for
the other two oligomers (L3 and L4) are described in
Example 7, below.

EXAMPLE 1

The 27 base oligomer containing a single mismatch
(L2 in Table 1) was kinased using ^{32}P-γ-ATP and hybrid-
ized to the + strand of M13mp9 clone II-16 DNA. M13mp9
clone II-16 DNA consists of bacteriophage M13mp9 DNA
into which has been cloned the 1.1 kb Pst I - BamHl
fragment of pBR322 oriented such that the plus strand
contains the Bam HI site at its 3' end. Bound oligomer
was then purified on a Sepharose® 4B column to remove
unbound oligomer from the reagent complex. Approxi-
mately 40% of the probe molecules were found to be
hybridized with labeled oligomer. About 250 ng of the
reagent complex were exposed to a 5-fold molar excess of
single-stranded competitor M13mp8 clone 20 DNA or non-
homologous M13mp 8 DNA, at 41°C in the presence of 2X
SSC for 20 min or 2 h. The plus strands of M13mp8 clone
20 DNA consists of bacteriophage M13mp8 DNA into which
has been cloned the 1.1 kb DNA strand complementary to

the pBR322 DNA present in M13mp9 clone II-16. Portions
of each sample were removed for gel electrophoresis and
autoradiography (data not shown, but they confirmed the
subsequent analysis). The remaining samples (5500-8000
cpm total activity) were applied to a 5 ml Sepharose® 4B
column. Three drop fractions were eluted in TE (10 mM
Tris, pH 8.0, 1 mM Na$_2$EDTA) and counted with scintil-
lant. The results are summarized in Table 2.

TABLE 2

Sample	Percent Total Counts	
	Early peak (bound oligomer)	Late peak (free oligomer)
1. original unchallenged reagent complex; 2h, 41°C	68%	32%
2. reagent complex + 5- fold molar excess of Ml3mp8 clone 20 DNA; 20 min., 41°C.	27	73
3. reagent complex + 5-fold molar excess of Ml3mp8 clone 20 DNA; 2h, 41°C.	5	95
4. reagent complex + 5-fold molar excess of non-homologous Ml3mp8 DNA; 2h, 41°C	71	29

EXAMPLE 2

Purified reagent comprised of single-stranded Ml3mp 9 clone II-16 DNA (See Example 1) and kinased 27-mer containing a single mismatch (labeled polynucleotide L2 in Table I, above) were prepared as described in Example 1. Competitive hybridizations were carried out at 50°C in the presence of 2X SSC for up to 1 h; samples were held on ice until subjected to electrophoresis on a 1.5 mm thick 5% (top) and 20% (bottom) stacked polyacrylamide gels. Electrophoresis was conducted for about 600 V-h and the stacked gel system was autoradiographed. Each sample contained 50 ng of the release tag - probe complex; Ml3mp8 clone 20 DNA served as the sample competitor DNA, while Ml3mp8 DNA and sheared calf thymus DNA were non-homologous additions. Table 3 summarizes the identity of samples loaded in individual polyacrylamide gel wells and the percent of free or unbound labeled oligomer. The percent oligomer values are derived from densitometric tracings of the autoradiogram taken for each gel lane.

TABLE 3

Lane	Sample Nucleic Acid	Time of Competitive Hybridization	Percent Free Oligomers
1.	orig. hybrid, unchallenged	—	8
2.	250 ng competitor DNA+1500 ng M13mp8	10 min	87
3.	250 ng competitor DNA+1500 ng M13mp8	1 h	97
4.	1500 ng M13mp8 DNA	1 h	3
5.	no competitor DNA	1 h	4
6.	2000 ng calf thymus DNA	1 h	3
7.	250 ng competitor DNA + 2000 ng calf thymus DNA	10 min	77
8.	250 ng competitor DNA + 2000 ng calf thymus DNA	1 h	98
9.	250 ng competitor DNA	10 min	69
10.	250 ng competitor DNA	1 h	98

The percent free oligomer values show that a sub-
stantial excess of M13mp8 DNA or complex calf thymus DNA
does not cause significant non-specific displacement of
the oligomer (labeled polynucleotide) and does not
inhibit the rapid displacement caused by competitor DNA
(the target nucleotide sequence).

EXAMPLE 3

The rate of displacement of a 27-base oligomer con-
taining a single mismatch (L2, Table 1) was compared to
that of a perfectly matched oligomer (L1, Table 1) bound
to the same probe polynucleotide sequence. Oligomers
were kinased using 32P-γ-ATP to specific activities of
$1-2 \times 10^8$ cpm/µg. Single-stranded probe DNA was prepared
by standard procedure from M13mp9 clone II-16 (see Exam-
ple 1). About 5µg of the probe polynucleotide were
hybridized with 45 ng of either perfect or single-
mismatch-containing oligomers (see Table 1) by heating
the mixture in the presence of 0.5 M NaCl to 65°C and
allowing the solution to cool slowly to room tempera-
ture. The hybridized oligomer (reagent complex) was
separated from free oligomer by elution from a Sepharose
4B column; approximately 50% of the probe molecules were
found to be hybridized to oligomer.

Competition hybridizations were performed at 60°C, in a final volume of 5μL, containing 2X SSC and 25 ng of the reagent complex. 25 ng of single-stranded M13mp8 clone 20 DNA (see Example 1) served as the competitor DNA having the target nucleotide sequence; non-homologous "competitor" DNA was from bacteriophage M13mp8 viral particles. Samples were placed on ice after incubation for desired periods, and subjected to electrophoresis in a 15% polyacrylamide gel. The area under peaks resulting from densitometric scans of an autoradiogram obtained from the gel were then quantitated. The fraction of total counts which appears as free oligomer (high mobility) and as reagent complex (low mobility) is tabulated below in Table 4 as a function of time of incubation.

TABLE 4

		% Total cpm			
		Low Mobility		High Mobility	
	Incubation Time	Perfect	Mismatch	Perfect	Mismatch
1.	1x competitor, 0 min	100	—	0	—
2.	5 min	87	—	12	—
3.	15 min	74	—	25	—
4.	30 min	60	42	39	58
5.	1 h	38	37	62	63
6.	2 h	42	23	57	77
7.	3 h	42	0	58	100
8.	No competitor, 3 h	100	81	0	18

While 50% of the perfectly matched oligomer is displaced at approximately 40 minutes, about 30 minutes is required to displace specifically 50% of the single mismatch-containing oligomer at the constant reaction temperature of 60°C.

EXAMPLE 4

An M13 clone containing approximately 1100 bases of an albumin cDNA clone as an insert (representing the target binding region of 1.1 kb in length) was prepared for use as the probe polynucleotide strand. An oligo-nucleotide 32 bases in length (L3 of Example 9) and com-

plementary to a 32-nucleotide sequence (the labeled polynucleotide binding region) near the middle of the 1.1 kb insert (target binding region) was end-labeled with phosphorous-32 (to be used as the labeled release tag polynucleotide) and hybridized to the M13 probe so as to form a reagent complex. Unhybridized 32-mer was removed by two passages through a 10 cm Sepharose® CL-4B column equilibrated in 0.3M NaCl, 10mM Tris and 0.1% SDS. The specific activity of the reagent complex was approximately 1000 cpm per ng of probe DNA. Approximately 1/3 of the probe strands had a labeled 32-mer polynucleotide bound. About 10 ng of the reagent complex was then combined in a 20µl volume of 1 M NaCl, 50mM Tris with one of the following:

A) 1000 ng sheared, boiled herring sperm (HS) DNA

B) 100 ng boiled, linearized plasmid Alb5/RII DNA containing albumin coding sequence on a 1400 nucleotide fragment (this coding sequence has a portion complementary to the 1.1 kb cloned insert in the above M13 clone and thus represents the target nucleotide sequence);

C) 100 ng of DNA from another M13 clone containing the opposite strand of albumin coding sequence than is present in the probe. This M13 DNA was digested with the restriction endonuclease HaeIII to linearize the DNA and to produce a linear fragment (2A/Hae III) containing a 1.72 kb albumin gene sequence which includes a contiguous sequence complementary to the 1.1 kb target binding region sequence).

D) 100 ng of DNA (4A/Hae III) as in C above except that the sequence of DNA coding for albumin that is present is the same as the target binding region in the probe complex.

E) No additional DNA, with incubation.

F) No additional DNA; no incubation.

Each of the above solutions was incubated at 50°C for two hours (except for F). Following incubation, each

sample was electrophoresed on a 2.5% agarose gel and the gel was autoradiographed. Several autoradiographs of varying exposure length were prepared. The results with the longest exposure (66 hrs with 2 intensifying screens) indicate that in the absence of true competitor DNA or in the presence of non-homologous competitor, no displacement of the release tag oligonucleotide occurs. However, in the presence of specific competitor, all (in case C) or almost all (in case B) of the release tag oligomer can be displaced. An exposure suitable for densitometry (98 hrs with no screen) was quantified by scanning densitometry. The peaks were carefully cut out of the gel scan, weighed, and the percentage of mass in the intact hybrid (I) and the displaced strand (D) peaks were determined; the results are summarized in the middle columns of Table 5. Following autoradiography, the gel was aligned with the autoradiogram and the regions of the gel containing the radioactivity were excised and counted by liquid scintillation counting. All samples were counted for 20 minutes. The background counts were 25 cpm. This background was subtracted from all samples resulting in the data in the righthand columns of Table 5. The quantification in Table 5 verifies the visual result seen and scored in the autoradiogram; that is, major amounts of displacement occurred only in those runs (cases B and C) in which the complex was contacted with DNA containing a target nucleotide sequence that could bind to the target binding region of the reagent complex.

Table 5
Quantitated Strand Displacement in Solution

			mass(ng)	%	cpm	%
A)	HS DNA	I	85.7	100	245	100
		D	0	0	0	0
B)	Alb 5/RII	I	25.6	32	60	41
		D	54.2	68	87	59
C)	2A/HaeIII	I	7.1	10	19	24
		D	66.0	90	61	76
D)	4A/HaeIII	I	73.5	100	250	100
		D	0	0	(- 2)	0
E)	NO DNA-2hr	I	70.1	100	218	100
		D	0	0	0	0
F)	NO DNA-0hr	I	86.4	100	200	100
		D	0	0	0	0

EXAMPLE 5
Displacement From A Supported Complex

The reagent complex described in Example 4 (10 ng) was adsorbed on Schleicher and Schuell NA45 paper (DEAE cellulose/nitrocellulose paper), air dried, and then irradiated with ultraviolet light (handheld short wavelength UV lamp held at a distance of 0.5 cm) for 0, 1, or 5 minutes. The filters were then incubated in a small volume of hybridization solution at 50°C for 4 hours. An aliquot of each solution phase was saved for electrophoresis, and the remaining solution removed and saved. Incubation of all filters was then continued overnight in fresh hybridization solution and aliquots were saved. Displacement reactions were carried out for 4 hours at 50°C with 100 ng of various sample nucleotide strands. An aliquot of each reaction solution phase was again saved for gel electrophoresis and the remainder was counted by liquid scintillation counting for two minutes (no background counting was done). All appropriate samples were electrophoresed on the same 2.5% agarose gel and the gel was autoradiographed. The results indicate that some of the composite reagent com-

plex and some 32-mer came off the filters in the first 4
hours preincubation. The UV treatment for 5 minutes
substantially decreased the amount of complex which came
off, although a small amount of 32-mer did still come
off. During the subsequent overnight incubation, very
few additional counts came off according to the results
of liquid spectrometry (Table 6). During the displace-
ment reactions, the 32-mer labeled polynucleotide was
specifically displaced only when the correct competing
DNA strand (containing the 1.1 kb target nucleotide
sequence) was present (Table 6, sample competitor 2A/H);
and essentially no displacement was detected with either
no competitor DNA (No Comp) or a large excess (1000 ng)
of non-specific herring sperm competitor DNA (HS DNA).

TABLE 6

	Competitor DNA	# cpm in reaction solution phase
0' UV	2A/H	1777
	No Comp	607
	HS DNA	714
1' UV	2A/H	2261
	No Comp	96
	HS DNA	154
5' UV	2A/H	2267
	No Comp	260
	HS DNA	272

EXAMPLE 6

A portion of pBR322 located between the Eco RI and
Sal I restriction sites (about 650 bases in length) was
cloned into M13mp8 or M13mp11. Using a
^{32}P-end labeled 27 nucleotide long oligonucleotide com-
plementary to a portion of this pBR322 sequence as
primer and the appropriate template chosen from the
above two clones, DNA synthesis was initiated on the
template and extended partially around the circle. The
DNA was digested with Eco RI and the resulting 375
nucleotide end-labeled fragment was purified by denatur-
ing agarose gel electrophoresis. This 375-mer was

annealed back to the pBR322/M13 probe strand to form a
reagent complex wherein the labeled polynucleotide was
bound to the end 375 nucleotides of a 650 nucleotide
target binding region. Unannealed 375-mer was removed
by a Sepharose® CL-4$_B$ column as described in Example
4. This reagent complex (10 ng) was then used in a
strand displacement reaction at 70°C in 0.4 M NaCl, 100
mM Tris. The sample competitor DNA used in each of the
five runs was:

A)   no DNA

B)   10 ng of circular M13mp11 into which had been
      cloned pBR322 DNA complementary to the target
      binding region over most of its 650 base length;
      this DNA thus contains the 650 base long target
      nucleotide sequence in an 7800 base long circle.

C)   100 ng of the same 7800-mer circular DNA as in B;

D)   100 ng of circular M13 into which had been cloned a
      pBR322 DNA strand identical to that found in the
      probe strand (and thus does not contain the target
      nucleotide sequence); and

E)   15 ng of a linearized, denatured 650 base long Eco
      RI/Sal I fragment of pBR322 (containing the 650
      base long target nucleotide sequence as a single-
      stranded linear DNA molecule as well as its 650
      base long complement).

After 0', 5', 15', 30' and 60', the displacement reac-
tions were stopped by quenching on ice; and at the end
of the experiment, the samples were electrophoresed on a
1.5% agarose gel and autoradiographed. There was a
slight contamination with unannealed 375-mer still
present (i.e., a background value of radioactivity was
seen at the location for 375 nucleotide length pieces in
Case A). No increase in this background was observed
over the 60' reaction, either with the negative controls
of no competitor DNA (Case A) or with the same strand as
sample competitor DNA (Case D). Specific displacement
was observed with the opposite strand as competitor
(Cases B and C) or with the denatured, linearized frag-

ment (Case E). The ability to quantitate the results of
this experiment are complicated by the existence of a
doublet of uncertain origin in all cases in the low
mobility gel region. Qualitatively, it appeared that
the approximately equimolar competitor:reagent ratio of
Case B yielded less complete and less rapid displacement
compared to the 10:1 molar ratios of Cases C and E (note
that the negative control Case D was also at a 10:1
molar ratio). Where displacement occurred in Cases B
and C, two new locations of peak radioactivity
occurred: one at a lower mobility (higher molecular
weight) than the initial complex (presumably an inter-
mediate structure such as in Figure 3B, but with both
probe P and competitor G being circular), the other at
the higher mobility (lower molecular weight) of dis-
placed 375-mer. The stability of the intermediate
structure is presumably due to the circular nature of
the probe strand and competitor. Case E does not show
the appearance of significant amounts of the lower
mobility peak because the challenger is linear in Case
E, but does show a second high mobility peak. This
second high mobility peak probably results from
rehybridization of the displaced labeled 375-mer to the
650 base-long complementary DNA strand present because
of the use of denatured double-stranded DNA as sample
competitor DNA in Run E.

EXAMPLE 7

Effect of Mismatches on the Rate of
Displacement from and Stability of Reagent Complex

In an experiment similar to Example 8, a perfectly matched albumin 32-mer and a 32-mer with 2 mismatches (resulting in G-T mispairing) were end labeled with phosphorus-32 and annealed to an albumin M13 clone as in Example 4. The sequence of the first of these two labeled polynucleotides is:

(Perfectly matched 32-mer): 3'end-CTACAAACGTT-TTTGATACGACTCCGTTTCCT 5'end (L3). In the mismatched 32-mer (L4), both underlined deoxycytidines of L3 were replaced by deoxythymidine. The stability of the reagent complexes was determined by melting experiments similar to those of Example 8 for 1,2 and 4 hours. The 32-mer without a mismatch was stable up to 50°C for 2 hours while the 32-mer with two mismatches was stable only up to 40°C for 2 hours.

Each of the reagent complexes was incubated with 100 ng of competing (2A/Hae III) M13-albumin DNA (see Example 4) in a 10 microliter volume for 5, 10, 20, 40, 80, and 120 minutes. The reaction temperatures studied were 40°C for both and also 50°C for the perfect match. The samples were analyzed by electrophoresis and autoradiography as described in Example 4. At 40°C the rate of displacement from the two reagent complexes was nearly identical. However, at 50°C, displacement from the perfectly matched reagent complex was significantly more rapid than from either of the reagent complexes at 40°C. This more rapid displacement is most likely an effect of the higher temperature of hybridization allow-able with the perfectly matched reagent complex, the higher temperature resulting in a more optimal rate of hybridization (See J. G. Wetmur and N. Davidson, _J. Mol. Biol._ 31, 349-370 (1968)).

EXAMPLE 8

Displacement of Release Tag from
Nitrocellulose-Bound Probe by Competitor DNA

Single-stranded M13mp9 clone II-16 DNA (see Example 1) was applied in dots (0.25µg each) to nitrocellulose filters, baked two hours at 80°C and hybridized to a 32p-end labeled 27-mer with a single base mismatch (L2, Table 1) at 50°C in 6X SSC plus 10X Denhardt's solution overnight. After extensive washes in 6X SSC (each dot retaining approximately 25,000 cpm), the dots were placed individually in 1.25 ml reaction mixtures containing 6X SSC plus 10X Denhardt's solution and either no competitor DNA, 2µg of M13mp8 clone 20 DNA (see Example 1), or 2µg of M13mp8 DNA (non-homologous competitor. Samples of 100µℓ were removed at intervals and counted by liquid scintillation spectrometry (Table 7 reports the results in counts per minutes in the 100 µl aliquots of the supernatant.

Table 7

Displacement time	No Competitor DNA	Competitor M13mp8 clone 20 DNA	ml3mp8 DNA
40 min.	40 (0)	73 (33)	41 (1)
60 min.	45 (5)	108 (68)	44 (4)
125 min.	56 (16)	195 (155)	59 (19)
180 min.	73 (33)	320 (280)	74 (34)
250 min.	94 (54)	424 (384)	92 (52)
395 min.	141 (101)	652 (612)	134 (94)

Values in brackets represent the raw (unbracketed) data after subtracting the 40 cpm machine background. These data show that the sample target nucleotide sequence in M13mp8 clone 20 DNA specifically displaces the radiolabeled release tag oligomer while sample DNA containing no target nucleotide sequence (i.e., M13mp8 DNA) does not displace oligomer above background levels.

EXAMPLE 9

Use of a Reagent Complex Having a
Residual Binding Region

A portion of plasmid pBR322 DNA located between the Eco RI and Sal I recognition sites was cloned into bacteriophage M13mp8 or M13mp11. A 32p-labeled 19 base primer complementary to sequences adjacent to the insert

in one of the M13 vectors (designated mp8pBR because M13mp8 was the cloning vector) was hybridized to the insert DNA of that clone and extended _in vitro._ with _E. coli_ DNA polymerase. The resulting partial double-stranded DNA was digested with the restriction enzyme Bam Hl and the 316 base long labeled polynucleotide product was then purified by denaturing agarose gel electrophoresis and hybridized to mp8pBR DNA. The resultant hybrids were purified away from unhybridized labeled polynucleotide by passage over two successive sepharose CL-4B columns.

The competitor for the displacement reaction was the opposite strand of the Eco R1-Sal I fragment cloned in bacteriophage M13mp11 (and therefore designated mp11pBR). This competitor DNA is fully complementary to the 275 bases of the probe which derive from the pBR322 DNA insert in the mp8pBR molecule. An additional 41 base pairs in the labeled polynucleotide are not comple-mentary to the competitor DNA, since they derive from sequences in the M13 vector. Thus, this probe has a tail (Residual Binding Region) which cannot be displaced by the competitor DNA, but must be melted off after a portion of the labeled polynucleotide has been displaced from the probe.

Displacement of this labeled polynucleotide was shown to occur in two steps, and to require a high reac-tion temperature. The release tag probe complex was hybridized to a 10-fold excess of circular or partially digested competitor at 70°C in 100mM NaCl, 100 mM Tris, pH 8.0 for 2 hours. Aliquots of the reaction mix were then heated for 5 or 15 minutes at either 80°C, 85°C or 90°C. The reactions were then quenched on ice and the samples were analyzed on an agarose gel by electro-phoresis followed by autoradiography.

After incubation with circular competitor DNA at 70°C, more than 90% of the reagent complex had become associated with target nucleotide sequence as indicated by a shift in the mobility of the reagent complex on the

gel. No such shift was seen when partially digested
competitor DNA was used. Because the competitor, DNA
associates with the probe polynucleotide without dis-
placing the labeled polynucleotide, hybridization of the
competitor and reagent complex results in a detectable
shift on the autoradiogram of labeled material towards a
position of lower mobility. Raising the temperature to
within the range of 80°C to 90°C for 5 or 15 minutes
resulted in the displacement of labeled polynucleotide
from the probe up to a maximum of 80% under the speci-
fied conditions.

An experiment similar to the above demonstrated
that the observed displacement events were specific.
Incubation of the same reagent complex described above
with a 10-fold molar excess of nonspecific competitor
M13mp7 DNA at 85°C in 100 mM NaCl, 100 mM Tris, pH 7.5
for 2 hours resulted in no displacement above background
levels of unbound labeled polynucleotide. The back-
ground is likely due to slight contamination of the
reagent complex with unhybridized labeled polynucleo-
tide. Incubation of this same reagent complex with an
equimolar or 10-fold molar excess of the specific com-
petitor mp11pBR at 85°C under similar conditions again
resulted in displacement of up to about 80% of the
labeled polynucleotide from the probe.

EXAMPLE 10
Release Tag Displacement from a Probe
Polynucleotide Tethered on a Solid Support

Single-stranded DNA from an albumin-M13 clone (4A
the probe of Example 4) was prepared and digested with
the restriction enzyme Hae III to produce small linear
DNA fragments containing human albumin CDNA sequences.
The linearized DNA was then tailed with a mixture of
nucleotides including a biotinylated derivative of dUTP
as follows. The solution for the tailing reaction con-
tained 200mM potassium cacodylate, 1mM 2-mercapto-
ethanol, 1mM cobalt chloride, 0.05mM dTTP, 0.05mM dCTP,
and 0.1mM biotinylated dUTP. Four units of terminal

deoxynucleotidyl transferase were added and the reaction
mixture incubated for 1 hour at 37°C. Control reactions
indicated that several hundred nucleotides were added to
each DNA fragment although not all tails contained a
biotinylated derivative (presumably due to a slower rate
of incorporation of the biotinylated derivative). The
tailed DNA was then annealed to 32p-labeled albumin 32-
mer L3 as described in Example 4. The annealing
reaction mixture (reagent complex) was passed over an
0.4 ml packed volume of avidin-agarose (Sigma) in
hybridization buffer (1 M NaCl, 50mM Tris, pH 8.0, 5X
Denhardt's solution). The flow-through was passed over
the column again and the column was then washed
extensively at room temperature with hybridization
buffer. The avidin-agarose beads containing the hybrid
were then removed from the column and distributed into
50µl aliquots in Eppendorf microfuge tubes. There were
approximately 30,000 cpm in each aliquot. Various
amounts of specific competitor used in Example 4
(between 0 and 200 ng) were added to each microfuge tube
and the samples were incubated at 50°C for 2 hours.
Following the reaction, the contents of the tubes were
transferred into small disposable columns (Bio-RadDispo-
columns). Following a brief centrifugation, the eluate
solution was recovered in an Eppendorf microfuge tube
and quantitated by Cerenkov counting. The eluted cpm
were as follows:

TABLE 8

Amount competitor DNA	Eluted cpm (% of input)
0	343 (1.2%)
0.5	454
2	553
10	1295
50	4822
100	8965
200	13190 (43%)

The 0.5 ng sample corresponds to roughly 1×10^8 competi-
tor DNA molecules detectable (111 cpm above background)

in 2 hours. A dose response curve drawn from this
experiment shows a high degree of linearity.

EXAMPLE 11

Hybrids between M13mp9 clone II-16 DNA and the
radiolabeled 27 base oligomer L1 were prepared by
heating 0.4 pmole M13mp9 clone II-16 DNA plus 2.0 pmole
oligomer in 36 μl 5X SSC (1X SSC = 0.15 M NaCl, 0.015
M sodium citrate) at 65°C for five minutes, then 50°C
for 30 minutes. The solution was fractionated over a
5.0 ml Sepharose® 4B column in 10mM Tris-HCl, pH 8.1,
1.0 mM Na_2EDTA and radioactivity for each fraction
determined by Cerenkov radiation counting. The hybrid
(reagent complex) fractions were pooled. The elution
profile indicated that more than 45% of the probe
strands (M13mp9 clone II-16 DNA) were hybridized to
oligomer (1100 cpm per 10 μl). Approximately 10 ng of
reagent complex were incubated for 0, 30, 60, 90, 120 or
150 minutes in 20 μl 2X SSC or 5X SSC at 50°C prior to
analysis by gel electrophoresis on 15% polyacrylamide
slab gels and autoradiography. The results from the
autoradiograms showed that there is no spontaneous
dissociation of the reagent complex at this temperature
$(T_m-18°)$ for at least 150 minutes under conditions which
mimic those for competitive strand displacement.

Samples of reagent complex (50 ng) were
subsequently mixed with 0% or 8% PEG (MW 6000) in 24
5X SSC and either no competitor DNA or 50 ng of
competitor M13mp8 clone 20 DNA and incubated at 50°C for
0, 30, 60 or 90 minutes before analysis by gel
electrophoresis in 15% polyacrylamide slab gels.
Densitometric scans of the resulting autoradiograms show
that strand displacement was barely detectable in the
absence of PEG, but significant in the presence of 8%
PEG even at the earliest observation time (Table 9).
PEG therefore has a significant and specific effect on
the rate of strand displacement at this concentration.

TABLE 9

Minutes of Incubation	8% PEG (MW 6000)	50 ng Competitor DNA	Percent Free Oligomer
0	-	-	26
	-	+	21
	+	-	23
	+	+	25
30	-	+	28
	-	+	29
	+	+	77
	+	+	79
60	-	+	19
	-	+	24
	+	+	94
	+	+	99
90	-	-	26
	-	+	33
	+	-	19
	+	+	90

EXAMPLE 12

Hybrids between M13mp9 clone II-16 DNA and radiolabeled 27 base oligomer were prepared by incubating 5 μ g M13mp9 clone II-16 DNA with 64.5 ng oligomer in 0.5 M NaCl, 10 mM Tris, pH 8.0, 1 mM Na_2EDTA at 65°C for 10 minutes in a waterbath and subsequently allowing the waterbath temperature to slow cool to 30°C. At this time the hybrid DNA was purified over a Sepharose® 4B column. The excluded hybrid DNA fractions were pooled and ethanol precipitated. Hybrid DNA was then resuspended to a concentration of 25 ng/μ l in 10 mM Tris, pH 8.0, 1 mM Na_2EDTA. About 25 ng of reagent complex were subsequently incubated at 55°C for 30 minutes or 3 hours in 10 μ l of 2X SSC containing 0, 8, 10 or 12 percent polyethylene glycol (PEG), MW6000. All samples were prepared in duplicate with half receiving no addition of competitor DNA and the other half receiving 25 ng competitor M13mp8 clone 20 DNA. Following incubation, the samples were subjected to

electrophoresis in 15% polyacrylamide slab gels. The
resulting gels were autoradiographed at -80°C with Kodak
X-omat® film and Dupont® Cronex® intensifying screen,
and scanned by densitometry.

After 30 minutes incubation at 55°C, the amount of
27 base oligomer DNA displaced from the probe DNA by
competitor DNA increased as the percent PEG, MW6000
solution present in the reaction increased from 0 to 12
percent (Table 10). PEG, MW6000 alone did not cause
dissociation of the oligomer DNA from the reagent
complex in the first thirty minutes in the absence of
Ml3mp8 clone 20 DNA (Table 10). After 3 hours
incubation at 55°C, the amount of 27 base oligomer DNA
found in the free form was still greater in the presence
of PEG, MW6000, at all polymer concentrations than in
its absence. The presence of PEG, MW6000, however, did
cause substantial non-specific dissociation of
hybridized 27 base oligomer DNA from the reagent complex
in the absence of competitor Ml3mp8 clone 20 DNA and
therefore conclusions about the specific effect of PEG
in promoting strand displacement cannot be drawn for
this lengthy period of incubation (see Example 6 and
Table 3 of U.S.S.N. 684,308).

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TABLE 10

Minutes of Incubation	Percent (w/v) PEG, MW6000	25ng Competitor DNA	Percent Free Oligomer
30	0	−	0
		+	0
	8	−	0
		+	68
	10	−	0
		+	100
	12	−	0
		+	100
180	0	−	0
		+	72
	8	−	47*
		+	100
	10	−	67*
		+	100
	12	−	32*
		+	100

*See Example 6 of U.S.S.N. 684,308 (and Example 16, below) for a discussion of these values.

The effective weight percent range of PEG, MW6000 on strand displacement reactions was further studied in another experiment. Hybrid DNA was made from ^{32}P-labelled 27 base oligomer (27-mer) and M13mp9 clone II-16 DNA which had been treated with the restriction enzyme HinfI. This enzyme cuts the M13mp9 DNA into several linear pieces without interrupting the region in M13mp9 clone II-16 containing the pBR322 DNA insert. The reaction mixture contained 0.8 pmoles of M13mp9 DNA and 6.8 pmoles of 27-mer in 77 µl of 5X SSC. The mixture was fractionated using a Sepharose® CL-4B column connected to a fraction collector and an elution buffer of 10 mM Tris-HCl, pH 8.1, 1.0mM Na$_2$EDTA. Radioactivity was determined for each fraction by Cerenkov counting. The faster moving peak was pooled as the hybrid. About 45% of the M13mp9 DNA was hybridized to labeled oligomer. About 20 ng of reagent complex in 24 µl of

5X SSC were incubated at 52°C for 90 minutes with 0 or
50 ng of competitor M13mp8 clone 20 DNA in the presence
of 0, 5, 12 or 19% PEG, MW6000. The samples were then
electrophoresed on a 15% polyacrylamide gel and the
resulting gels subjected to autoradiography.
Densitometric scans of the autoradiograms show that 5%
PEG is ineffective in influencing the extent of strand
displacement (Table 11) under these conditions while PEG
concentrations of 12% and 19% significantly enhance
strand displacement in a specific fashion.

Table 11

Percent (w/v) PEG, MW 6000	Competitor DNA Present	Percent Free Oligomer
0	−	38
	+	47
5	−	37
	+	47
12	−	42
	+	87
19	−	37
	+	100

The significant weight percent range of PEG, MW6000
that enhanced strand displacement is thus at least 8-19%
under these particular conditions (see 8% in Table
10). Examples below show effects at 2.5% and 5.0% PEG
under different buffer conditions.

EXAMPLE 13

About 25 ng reagent complex DNA were prepared as
described in Example 13 and were incubated at 55°C for
60 minutes in 10 µ l of 2X SSC containing 10% (w/v)
polyethylene glycol of MW 200, 400, 1000, 3350, 6000 or
20,000 daltons. Samples were prepared in duplicate with
half of the samples receiving 25 ng competitor M13mp8
clone 20 DNA and half receiving none. Following
incubation for 60 minutes, all samples were subjected to
electrophoresis in 15% polyacrylamide slab gels. The
resulting gels were autoradiographed at -80°C with
Kodak® X-omat® film and a Dupont® Cronex® intensifying

screen, and the autoradiograms were evaluated by
densitometric scanning. Polyethylene glycols of
molecular weights 3350, 6000 and 20,000 were more
effective in enhancing competitive displacement of
hybridized oligomer DNA than the polyethylene glycols of
lower molecular weights (Table 12).

TABLE 12

Polyethylene glycol	Percent Free Oligomer	
MW	– competitor DNA	+ Competitor DNA
200	0	64
400	0	60
1,000	0	59
3,350	0	100
6,000	0	100
20,000	0	79
Incubated Control, no Polyethylene Glycol	0	24

EXAMPLE 14

Hybrids between M13mp9 clone II-16 DNA and the
radiolabeled 27 base oligomer L1 were prepared. About
20 ng of reagent complex in 24 ul of 5X SSC were
incubated in the absence or presence of 10% polyethylene
glycol (PEG), MW6000 at 52°C for 90 minutes with
addition of 0, 2.0, 5.0, 20, 50, or 100 ng of competitor
M13mp8 clone 20 DNA. All samples were immediately
electrophoresed on 15% polyacrylamide slab gels and the
resulting gels subjected to autoradiography.
Densitometric scans of the autoradiograms show that the
sensitivity of strand displacement reactions is enhanced
by the inclusion of 10% PEG, MW6000 (Table 13).

TABLE 13

Inclusion of 10% PEG, MW6000	Amount of Competitor DNA (in ng)	Percent Free Oligomer
-	0	38
-	5.0	42
-	20	47
-	50	47
-	100	57
+	0	42
+	2.0	51
+	5.0	83
+	20	93
+	50	87
+	100	90

In a related experiment using a more highly puri-
fied reagent complex, reagent complex hybrids were
prepared and purified as described in Example 12. About
25 ng hybrid DNA were then incubated in the presence of
10 µl 2X SSC and either 2.5, 6.25, 12.5, 25 or 50 ng
competitor M13mp8 clone 20 DNA at 55°C for 60 minutes,
resulting in ratios of reagent complex DNA:competitor
DNA of 1:0.1, 1:0.25, 1:0.5, 1:1.0 or 1:2.0,
respectively. The samples were prepared in duplicate
with half receiving 10% PEG, MW 6000 and half receiving
none. Following incubation, the samples were subjected
to electrophoresis on 15% polyacrylamide slab gels and
the resulting gels were autoradiographed at -80°C with
Kodak X-omat film and a Dupont Cronex intensifying
screen. Densitometric scans of the resulting
autoradiogram demonstrate that, at all reagent complex
DNA:competitor DNA ratios, specific disassociation of
hybridized 27 base oligomer from probe DNA by competitor
DNA was greater in the presence of 10% PEG, MW6000, than
in its absence (Table 14).

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TABLE 14

Amount of reagent complex DNA (in ng)	Amount of competitor DNA (in ng)	% PEG, MW6000	Percent Free Oligomer
25	2.5	0	0
		10	17
25	6.25	0	20
		10	47
25	12.5	0	13
		10	83
25	25	0	32
		10	100
25	50	0	41
		10	100

Example 15

Control experiments were carried out to examine non-specific polyethylene glycol (PEG) interactions with reagent complex hybrids for strand displacement. In an initial experiment, hybrids between M13mp9 clone II-16 DNA and the 27 base perfectly matched oligomer L1 were prepared and purified as described in Example 13. About 225 ng hybrid DNA were incubated at 55°C in 90 ul of 2X SSC containing 10% (w/v) PEG, MW 6000. Aliquots of 10 were removed at the start of the experiment and at 30, 90 and 150 minutes. The aliquots were subjected to electrophoresis on 15% polyacrylamide slab gels, the resulting gels were autoradiographed at -80°C and the autoradiograms obtained were evaluated by densitometric scanning using a Shimadzu CS930 plate scanner. The reagent complex was stable as a hybrid DNA under these incubation conditions for up to 2 1/2 hours.

This experiment was terminated at 2.5 hours. In other experiments where mixtures with 10% PEG went for 3 hours (including the experimental values shown by asterisks in Table 10, above), non-specific dissociation of reagent complex hybrids was observed at 3 hours. Rather than attempt to understand this anomalous result, experiments conducted afterwards by the same group were

terminated in less than 10 hours.

In a related experiment to study potential aggregation of oligomer and polyethylene glycol (PEG), MW 6000, 0.15 pmole of radiolabeled oligomer was incubated in 20 ul 2X SSC or 5X SSC for 30, 90 or 150 minutes with or without 10% (w/v) PEG 6000 and then analyzed by gel electrophoresis and autoradiography and densitometric scanning. The results demonstrated that oligomer does not spontaneously associate with PEG 6000 and remove the possibility that observed decreases in free oligomer from unpurified reagent complex preparations are artifactual.

Example 16

An 840 base pair Eco Rl-Bgl II restriction fragment containing the first 262 codons of the human serum albumin gene was subcloned into the single-stranded phage vector M13mp9, generating the clone designated M13mp9-albumin. (See Lawn et al., *Nucleic Acid Research* vol. 9, pp. 6103-6114 (1981) for the DNA sequence of the human albumin gene). Also inserted into this vector was the Eco RI fragment containing the polylinker from M13mp7. This polylinker sequence, inserted adjacent to the albumin sequence at the Eco RI site, is capable of forming a double-stranded hairpin structure in the otherwise single-stranded molecule. Cleavage of such a molecule with the enzyme Bam HI results in a linear and full length single-stranded molecule. An end-labeled polynucleotide was kinased using ^{32}P-γ -ATP prepared by hybridizing a 14 base long oligonucleotide (5' end-GATGCACACAAGAG-3' end) to sequences within the albumin insert, and extending it *in vitro* using *E. coli* DNA polymerase. The resulting DNA molecules were then digested with Pvu II, which cuts once in the human albumin insert. The final labeled polynucleotide, a 175 base long labeled and single stranded molecule, was then purified by electrophoresis on a denaturing agarose gel. The purified labeled polynucleotide was hybridized to the above M13mp9-albumin strand which serves as the

probe polynucleotide; the probe used was either circular
or had been linearized with Bam HI. Unhybridized
labeled polynucleotide was removed from the reagent com-
plex by passage over two successive Sepharose® CL-4B
columns.

Model competitor DNAs used in displacement experi-
ments were either the complementary DNA strand to the
human albumin sequence of the M13mp9-albumin clone
inserted in an M13mp8 vector (and hereafter designated
as M13mp8-albumin), or a denatured double-stranded 840
base pair Eco RI-Bgl II fragment which is colinear with
the insert in the M13mp9-albumin clone.

Displacements were carried out by incubating of
approximately 10 ng of reagent complex with 100 ng of
analyte DNA in 0.2 M NaCl, 100 mM Tris, pH 8.0, at 85°C
for 5, 15, 30 and 60 minutes. At appropriate times,
samples were placed on ice to stop the reaction, and
then all samples were run on a 1.5% agarose gel to
assess displacement efficiency.

In the absence of analyte, no melting of the probe-
template complex was observed at 5, 15 or 30 minutes,
although at 60 minutes approximately 10% of the probe
was melted at this temperature. The addition of a
tenfold excess of analyte to the displacement reaction
resulted in the displacement of approximately 50% of the
probe at 15 minutes, 75% at 30 minutes and 95% at 60
minutes. The addition of 2.5% (w/v) PEG, MW 6000 to the
reaction mix enhanced the displacement rate two-fold;
approximately 95% displacement occurred during 30
minutes incubation. The addition of 5% (w/v) PEG, MW
6000 to the reaction further increased the rate of the
displacement reaction significantly: displacement was
essentially complete within 15 minutes. The addition of
10% (w/v) PEG, MW 6000 also apparently enhanced the rate
of the displacement still further, although the extent
of this enhancement could not be accurately estimated
from the autoradiograph due to a gel artifact frequently
observed with high concentrations of PEG at high

temperature in this buffer system. Although this experiment did not include controls of displacement complex without competitor, but in the presence of PEG, another experiment did include such a control. In that experiment, incubation under similar conditions to those of this Example with 10% (w/v) PEG, MW 6000 caused no detectable non-specific melting (dissociation) of the reagent complex in up to 60 minutes of incubation. Thus, increasing concentrations of PEG (at 85°C) appear to specifically increase the rate of the hybridization-strand displacement.

Example 17

A reagent complex was prepared as in Example 4 having a 1.1 kb albumin insert within M13 bacteriophage as the target binding region and an end-labeled 32-mer oligomer as the labeled polynucleotide. 10 ng of this reagent complex was combined with either:

a) 100 ng of boiled plasmid pAlb5 digested with Pst I and Bgl II containing an albumin coding sequence complementary to part of the albumin insert in the probe (including the 32 bases bound to radiolabeled 32-mer); or

b) 100 ng of boiled plasmid pUC8 digested with TaqI (this sequence is non-complementary and thus serves as a control).

The samples were combined in a 10 ul volume containing 50mM Tris, 1M NaCl and either 0, 5, 10, 15 or 20% PEG, MW 6000. Following incubations for 10 minutes at 50°C, the samples were immediately quenched in a dry ice/ethanol bath, thawed on ice and electrophoresed on a 2.5% agarose gel and autoradiographed. The amounts of displaced polynucleotides were estimated visually, as shown in Table 15. The results show no detectable displacement in the absence of the specific analyte at any PEG concentration and a significant enhancement in the amount of displaced labeled polynucleotide in 10 minutes under these conditions in the presence of the specific analyte when 5 or 10% (w/v) PEG, MW 6000 was

also present.

TABLE 15

% PEG, MW 6000	Non-Specific Competitor	Specific Competitor
0	ND	10%
5	ND	40%
10	ND	50%
15	ND	20%
20	ND	5%

ND = not detectable, i.e. < 2%

WHAT IS CLAIMED IS:

1. A method for determining the presence of a predetermined target nucleotide sequence in the nucleic acid of a biological sample which comprises the steps:

(a) providing a reagent complex of (i) a probe polynucleotide which is capable of base pair binding via hydrogen bonds of purine/pyrimidine bases to the target nucleotide sequence, and (ii) a labeled polynucleotide which is bound by base pair binding via hydrogen bonds of purine/pyrimidine base pairs to the probe polynucleotide ·in a region of the probe polynucleotide at least partially coextensive with the region in which the probe polynucleotide is capable of binding to the target nucleotide sequence;

(b) contacting the reagent complex with a sample under conditions in which the target nucleotide sequence, if present, binds to the probe polynucleotide and displaces labeled polynucleotide from the.complex; and

(c) determining the presence of labeled polynucleotide displaced from the reagent complex.

2. The method of claim 1 wherein the probe polynucleotide contains a target binding region capable of base pair binding to the target nucleotide sequence and a labeled polynucleotide binding region bound to bases of the labeled polynucleotide in the complex, and wherein the labeled polynucleotide binding region is contained within the target binding region.

3. The method of claim 1 or 2 wherein the labeled polynucleotide binding region is about 20 to about 500 nucleotides in length.

4. The method of any previous claim wherein the portion of the target binding region that is not part of the labeled polynucleotide region is at least about 100 nucleotides in length, and is at least as large as the labeled polynucleotide binding region.

5. The method of any previous claim wherein the probe polynucleotide contains a target binding region

capable of base pair binding to a sample target
nucleotide sequence and contains a labeled
polynucleotide binding region bound by purine/pyrimidine
base pairing to the labeled polynucleotide of at least
about 15 nucleotides; at least some of the nucleotides
of the labeled polynucleotide binding region being
included in the target binding region; and no more than
about 15 nucleotides of the labeled polynucleotide
binding region being outside the target binding region.

6. The method of any previous claim wherein the
probe polynucleotide is immobilized to a solid support
in the complex.

7. The method of claim 6 wherein the determining
step (c) comprises:

(c1) separating a first phase containing immobil-
ized probe polynucleotide from a second phase comprising
displaced labeled polynucleotide; and

(c2) determining the presence of labeled poly-
nucleotide in the second phase.

8. The method of any of claims 1-5 wherein the
reagent complex is free in solution during the
contacting step (b).

9. The method of claim 8 wherein the determining
step (c) comprises:

(c1) separating the reagent complex remaining from
a portion of the reaction solution after the contacting
step (b), and

(c2) determining the presence of any displaced
labeled polynucleotide in a solution phase after separa-
tion.

10. The method of any previous claim wherein the
base pairing between the labeled polynucleotide and the
probe polynucleotide is interrupted by at least one
mismatched pair.

11. The method of any previous claim wherein the
labeled polynucleotide is a polynucleotide bonded to an
enzyme.

12. The method of any previous claim wherein the

target nucleotide sequence is DNA.

13. The method of any previous claim wherein the probe polynucleotide is DNA.

14. A method for determining the presence of a predetermined target nucleotide sequence in the nucleic acid of a biological sample which comprises the steps:

(a) providing a reagent complex of (i) a probe polynucleotide which is capable of base pair binding via hydrogen bonds of purine/pyrimidine bases to the target nucleotide sequence, and (ii) a labeled polynucleotide which is bound by base pair binding via hydrogen bonds of purine/pyrimidine base pairs to the probe polynucleotide in a region of the probe polynucleotide at least partially coextensive with the region in which the probe polynucleotide is capable of binding to the target nucleotide sequence;

(b) contacting the reagent complex with a sample under conditions in which the target nucleotide sequence, if present, binds to the probe polynucleotide and displaces labeled polynucleotide from the complex; and

(c) determining the presence of labeled poly-nucleotide remaining in the reagent complex.

15. The method of any previous claim wherein the conducting step (b) is conducted in the presence of a polyether polymer under conditions in which the target nucleotide sequence, if present, binds to the probe polynucleotide and displaces labeled polynucleotide from the complex, the polyether polymeric agent being of a molecular weight and at a concentration sufficient to increase the rate of appearance of displaced labeled polynucleotide.

16. The method of claim 15 wherein the polyether polymer is a poly(alkylene oxide) of the formula $H(O-R)_n$ wherein R is an alkylene moiety of 1-6 carbons and n is an integer, on a weight average molecular weight basis, of about 35 to about 690.

17. The method of claim 15 wherein the polyether

polymer is poly(ehtylene oxide).

18. The method of claim 17 wherein the
poly(ethylene oxide) is of weight average molecular
weight of at least about 1500.

19. The method of any of claims 1-14 wherein the
contacting step (b) is conducted in the presence of a
volume excluding inert polymeric agent which is non-
ionic or anionic of sufficient molecular weight and
amount relative to the reagent complex to increase the
rate of appearance of displaced labeled polynucleotide.

20. A diagnostic reagent for determining the
presence of a predetermined target nucleotide sequence
in the nucleic acid of a biological sample comprising:

(i) a probe polynucleotide which is capable of base
pair binding via hydrogen bonds of purine/pyrimidine
base pairs to the target nucleotide sequence, and

(ii) a labeled polynucleotide which is bound by
base pair binding via hydrogen bonds of
purine/pyrimidine base pairs to the probe polynucleotide
in a region of the probe polynucleotide at least
partially coextensive with the region in which the probe
polynucleotide is capable of base pair binding to the
target nucleotide sequence;

the potential base pair binding between the target
nucleotide sequence and the probe polynucleotide being
capable of displacing the labeled polynucleotide from
the reagent complex.

21. The diagnostic reagent of claim 20 wherein the
probe polynucleotide contains a target binding region
capable of base pair binding to the target nucleotide
sequence and a labeled polynucleotide binding region
bound by purine/pyrimidine base pairing to bases of the
labeled polynucleotide in the complex, and wherein the
labeled polynucleotide binding region is contained
within the target binding region.

22. The diagnostic reagent of claim 20 or 21
wherein the labeled polynucleotide binding region is
about 20 to about 500 nucleotides in length.

23. The diagnostic reagent of claim 20, 21 or 22 wherein the probe polynucleotide is immobilized to a solid support in the reagent complex.

24. The diagnostic reagent of claim 20, 21 or 22 completely free in solution.

25. The diagnostic reagent of claim 24 wherein the probe polynucleotide contains an attached moiety for affinity separation of reagent complex from displaced labeled polynucleotide.

26. A diagnostic kit for determining the presence of a predetermined target nucleotide sequence in the nucleic acid of a biological sample comprising:

(a) the reagent complex of any of claims 20-25, and

(b) a polyether polymer of sufficient molecular weight and amount relative to the reagent complex to increase the rate of appearance of displaced labeled polynucleotide.

27. A diagnostic kit for determining the presence of a predetermined target nucleotide sequence in the nucleic acid of biological smaple comprising:

(a) the reagent complex of any of claims 20-25, and

(b) a volume excluding inert polymeric agent which is non-ionic or anionic of sufficient molecular weight and amount relative to the reagent complex to increase the rate of appearance of displaced labeled polynucleotide.

FIG. IA

FIG. IB

FIG. IC

FIG.ID

FIG.1E

FIG.1F

FIG.1G

FIG. 2

FIG.4
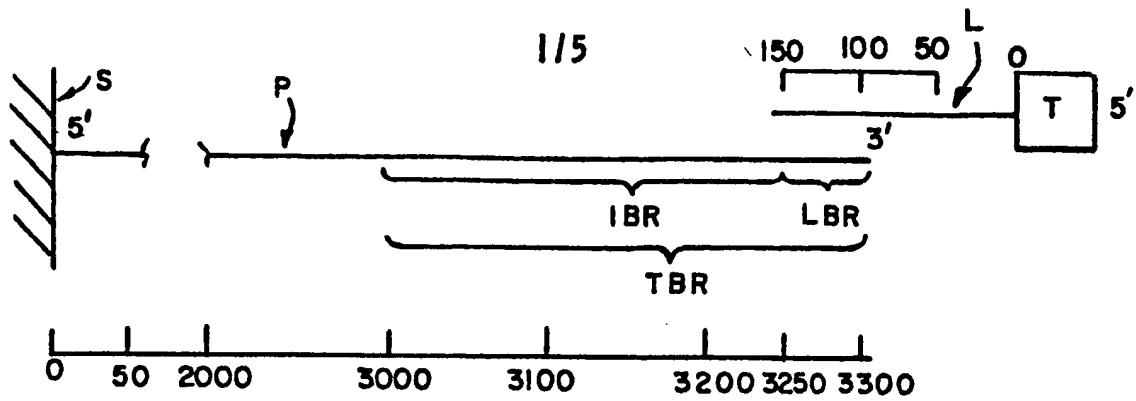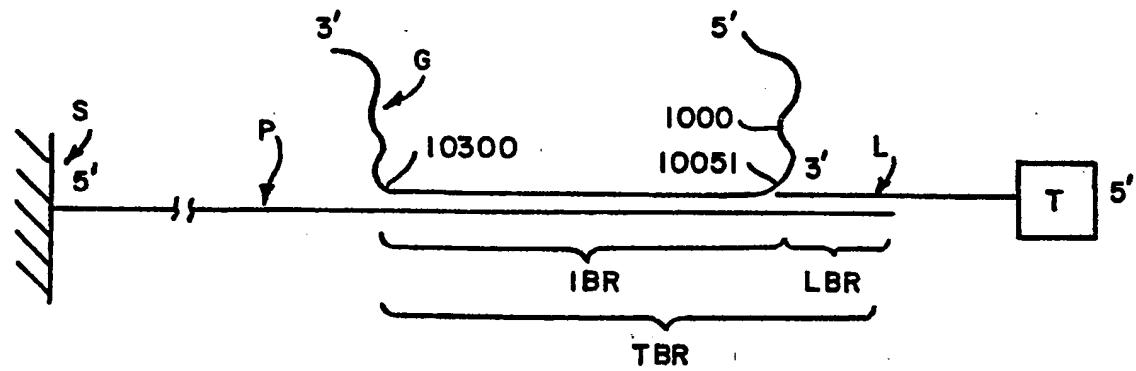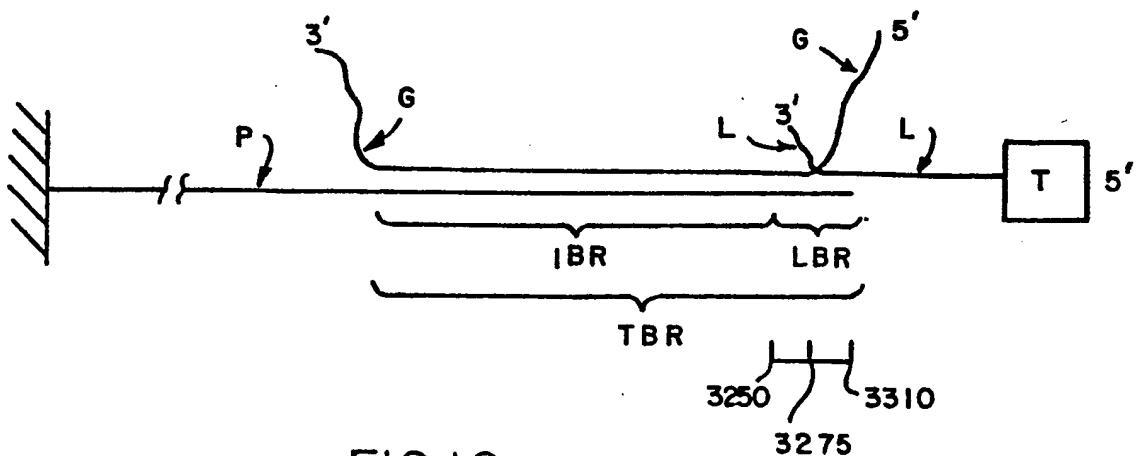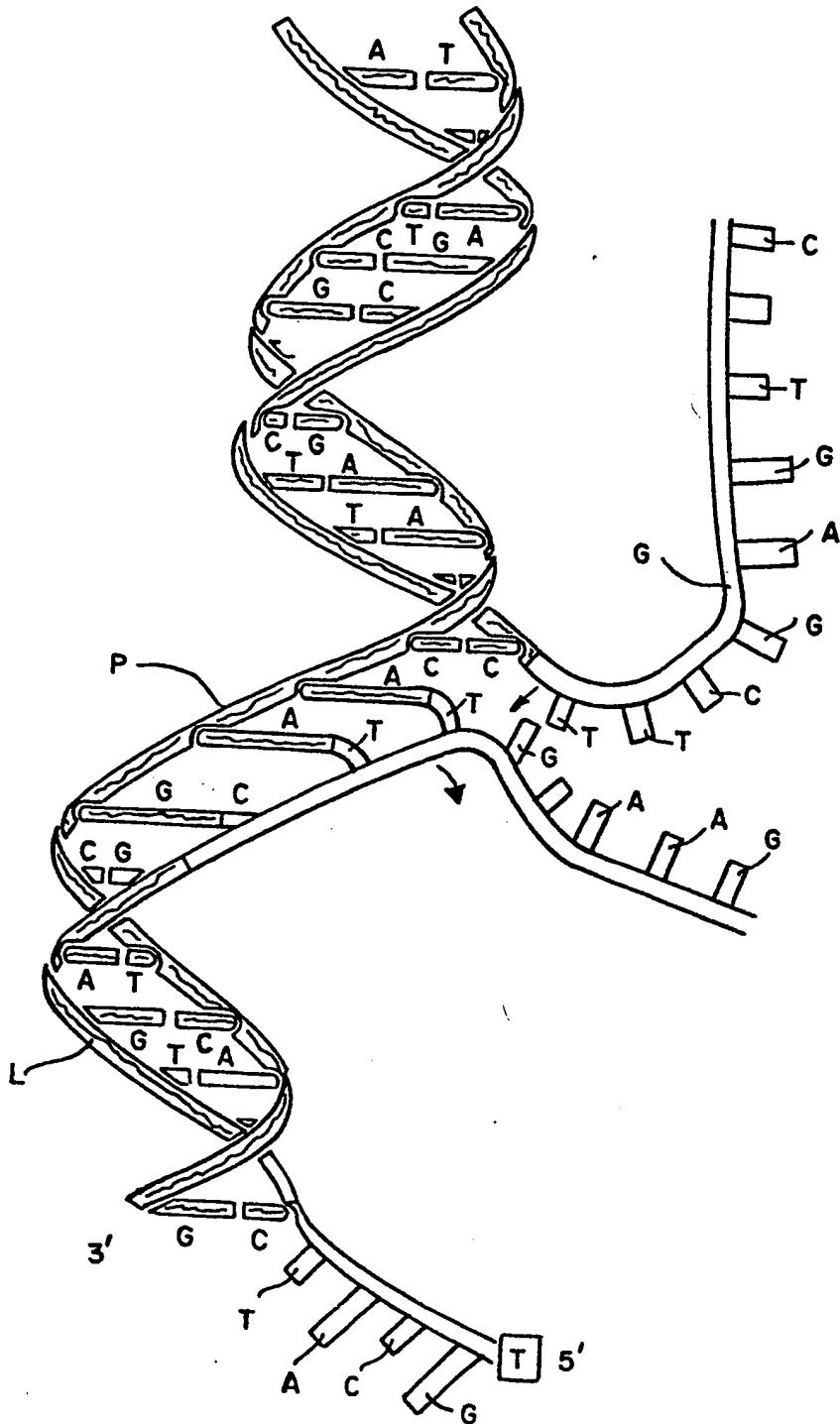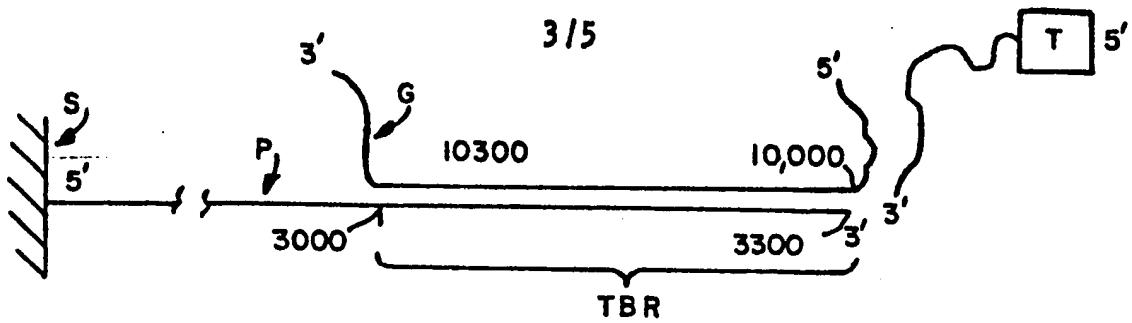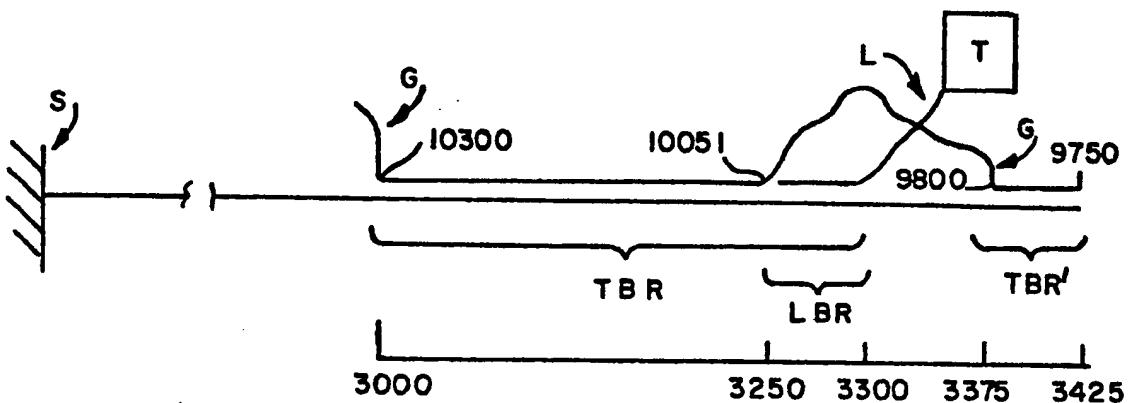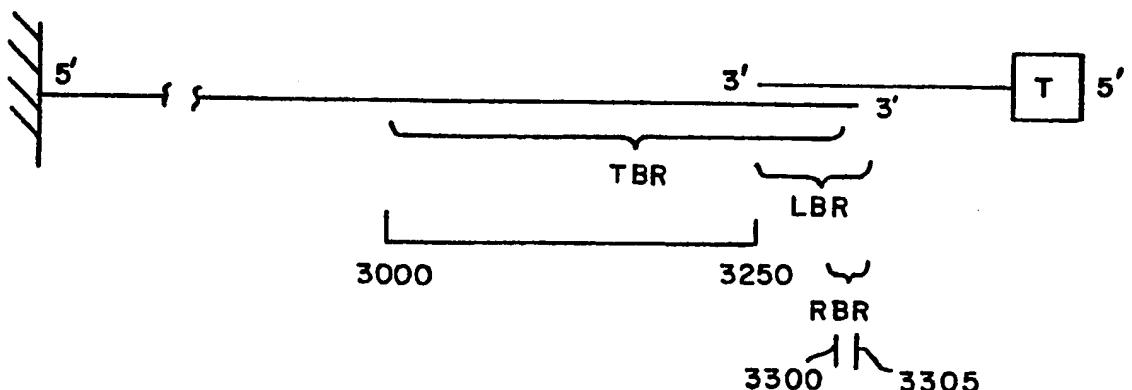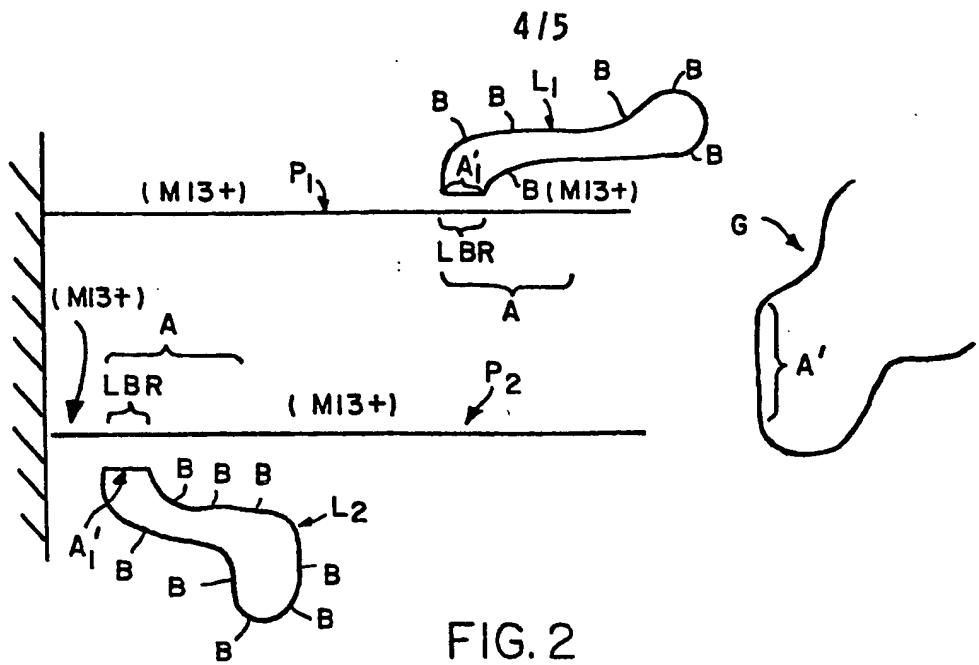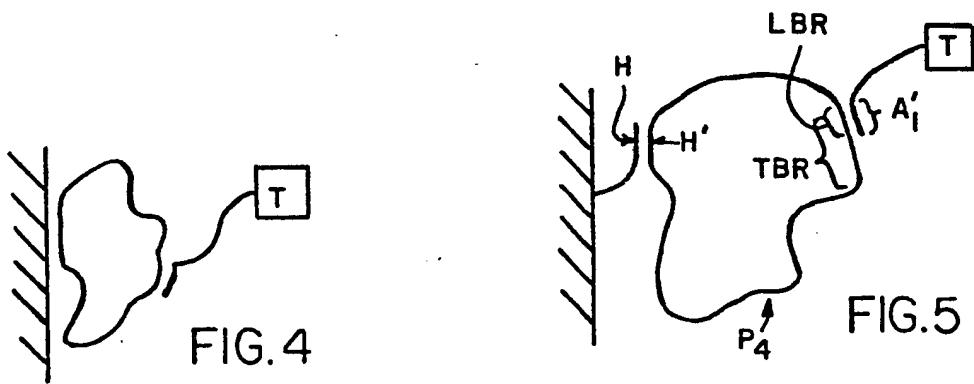
FIG.5
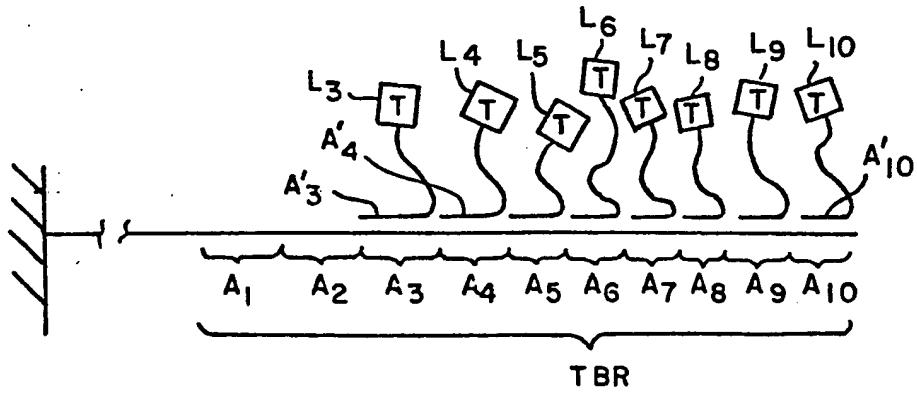
FIG.6
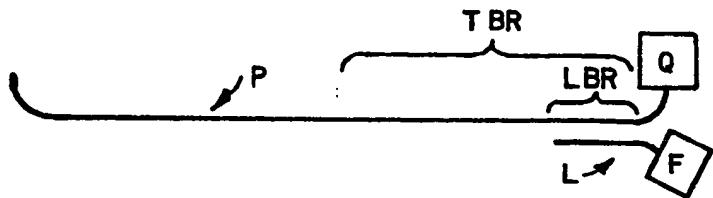
FIG.3A
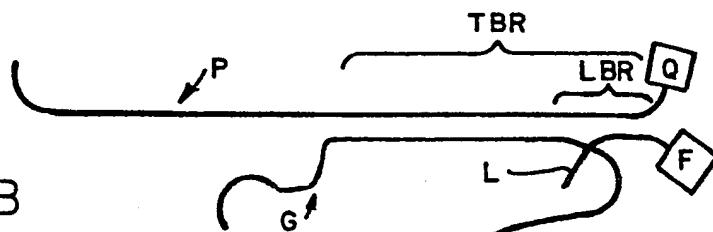
FIG.3B
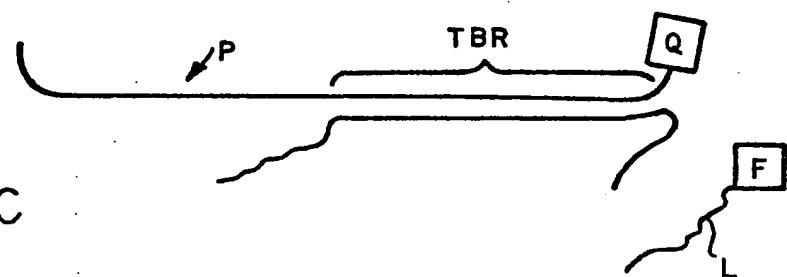
FIG.3C
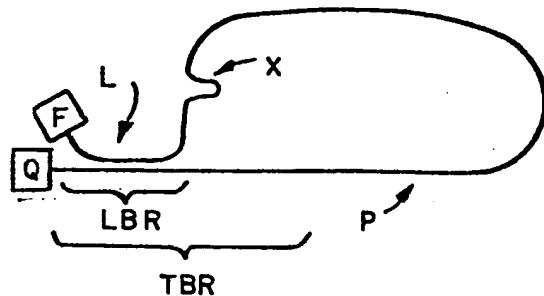
FIG.3D

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int Cl 4)
A	US-A-4 358 535 (S. FALKOW et al.) * Column 2, line 20 - column 4, line 23 *	1,2,20,26	C 12 Q 1/68
A,D	US-A-4 395 486 (L.B. WILSON et al.) * Column 2, line 43 - column 3, line 8; column 4, line 55 - column 5, line 37 *	1,2,20,26	
A	EP-A-0 070 685 (STANDARD OIL CO.) * Page 4, line 5 - page 6, line 8; pages 16,17; claims 1-5 *	1,2,20,26	
A	US-A-4 302 204 (G.M. WAHL) * Column 2, lines 41-45; column 9,10; example VIII *	1,2,15-20,26,27	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 Q 1/00 G 01 N 33/00 C 12 N 15/00
P,A	EP-A-0 130 515 (MOLECULAR DIAGNOSTICS) * Page 1, line 19 - page 3, line 9; page 3, line 24 - page 4, line 25; pages 22,23; claims 1-5 *	1,2,20,26	
P,A	EP-A-0 124 221 (A.D.B. MALCOLM) * Page 5, line 21 - page 7, line 29 *	1,2,20,26	

The present search report has been drawn up for all claims

Place of search BERLIN	Date of completion of the search 31-07-1985	Examiner GREEN C.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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